The remarkable process known as apoptosis is responsible for cell death in development, normal tissue turnover, atrophy induced by endocrine and other stimuli, negative selection in the immune system, and a substantial proportion of T-cell killing. It also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia or viral infection. It is a major factor in the cell kinetics of tumors, both growing and regressing. Many cancer therapeutic agents exert their effects through initiation of apoptosis, and even the process of carcinogenesis itself seems sometimes to depend upon a selective, critical failure of apoptosis that permits the survival of cells after mutagenic DNA damage.\[1\text{-}3]\]

Apoptosis can be highly influenced by the local microenvironment of a tumor and represents only one form of cell death that can contribute to the inactivation of tumor cells. The importance of other non-apoptotic pathways are frequently overlooked with respect to their role in preventing cancer and, even more so, in determining treatment sensitivity.

**Methods for studying apoptosis**

A number of methods have now been developed to study apoptosis in cell populations. The two key apoptotic events in the cell: Apoptosis and cell mediated cytotoxicity are characterized by cleavage of the genomic DNA into discrete fragments prior to membrane disintegration. Because DNA cleavage is a hallmark for apoptosis, assays which measure prelytic DNA fragmentation are especially attractive for the determination of apoptotic cell death. The DNA fragments may be assayed in either of two ways: As “ladders” (with the 180 bp multiples as “rungs” of the ladder) derived from populations of cells, e.g., with the Apoptotic DNA Ladder Kit. By quantification of histone complexed DNA fragments with an ELISA.\[2\text{-}3]\]

Further, the proteases were involved in the early stages of apoptosis. The appearance of these caspases sets off a cascade of events that disable a multitude of cell functions. Caspase activation can be analyzed in

**Key words:** Apoptosis, cancer therapy, clonogenic survival, senescence

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DOI: 10.4103/0971-6866.92081
different ways: By an *in vitro* enzyme assay. Activity of a specific caspase, for instance caspase 3, can be determined in cellular lysates by capturing of the caspase and measuring proteolytic cleavage of a suitable substrate.[2,3]

Second method is by detection of cleavage of an *in vivo* caspase substrate. For instance caspase 3 is activated during early stages. Its substrate PARP (Poly-ADP-Ribose-Polymerase) and the cleaved fragments can be detected with the anti PARP antibody.[1-3]

**Apoptosis during Cancer development and treatment**

Cell death mechanisms can contribute to cancer development and treatment response in largely varying degrees and the loss or down regulation of cell death pathways clearly occurs during cancer development, not only in the case of apoptosis but also in other mechanisms of cell death. A carefully regulated balance is needed between cell proliferation and cell death to allow proliferation to exceed cell death and consequently to the development of a tumor. Selection against apoptosis may be especially important, as many oncogenes not only promote proliferation but also sensitize the cell to death by apoptosis.[2]

There are certainly compelling experiments that have clearly demonstrated that apoptosis can influence tumor response. This has been perhaps most elegantly studied in the E-myc lymphoma model developed by Lowe and colleagues.[3]

Tumors arising because of the presence of myc activation in the B-cell lineages are highly influenced by secondary pathways that affect apoptosis. This model clearly demonstrates that apoptosis acts as a barrier to cancer development and also provided evidence that apoptosis is important for cancer therapy.[4]

The model described an extreme sensitivity to apoptosis because of two distinct factors. First, the cells of lymphoid origin generally show a much higher propensity to die by apoptosis.[5,6] Second, overexpression of myc also results in an increased sensitivity toward apoptosis.[7] Indeed, Brown and Wilson concluded that “there is little or no support that apoptosis, and the genes govern it, determine the response to therapy”.[8] In contrast to the lymphoma model, they found that the propensity to undergo apoptosis in human tumors of epithelial origin played no role in predicting treatment sensitivity.[1-3]

The concept that selection of cells with resistance to apoptosis during carcinogenesis results in a co-selection of cells that will be resistant to treatment has become a persuasive one in the research community. It is important to evaluate all potential forms of cell death that may contribute to treatment response.[9,10] Furthermore, for each type of cell death, it is critical to consider both the kinetics of cell death and its dose-response relationship with the treatment.[2,3]

**Survival in relation to dose response and cell kinetics**

In many cells, several forms of cell death may be possible in response to cancer treatment. The form of cell death that actually inactivates any particular cell is obviously the one that occurs first, that with the fastest kinetics. In cells that retain considerable apoptotic sensitivity, the most rapid form of cell death is often apoptosis, even though it may not be the most sensitive. This is particularly true when treating cells with high doses of a DNA-damaging agent. However, this does not imply that other mechanisms of cell death would not have occurred if given the opportunity [Figure 1].[2,3]

![Figure 1: Importance of dose-response relationships for different modes of cell death. Shown are hypothetical dose-response curves for death (upper frames) and survival (lower frames) in response to an anti-cancer agent. The dose-response relationships for overall (solid), apoptosis only (short dash), and non-apoptosis mechanisms (long dash) are shown separately.](image)
Differences in the rate of cell death have complicated the importance of apoptosis for two distinct reasons. The first is because many assays are biased to detect rapid changes in cell number. Rapid forms of cell death are thus more easily detected by these types of assays. The second is because the most rapid form of cell death can mask other forms of death.\[^{[3]}\]

Consequently, if multiple forms of cell death are activated in the same cell, the one that is manifested first will be the only one observed. The most rapid form of death will not necessarily be the most important (sensitive). Interestingly, studies with the E-myc model of lymphoma also provide one of the best examples of how disabling one cell death pathway can reveal another that is equally as sensitive.\[^{[1-3,11]}\] In this model, the development of lymphoma (time to onset/aggressiveness) is very strongly influenced by loss of apoptotic sensitivity. Loss of the INK4A/ARF locus reduces apoptosis and accelerates tumor development in this model. This locus encodes two tumor suppressors: ARF, which is responsible for activating p53 in response to oncogene activation, and INK4A, which encodes the p16 CDK inhibitor. Thus, the loss in apoptotic sensitivity following deletion of the entire INK4A/ARF locus is due entirely to ARF. Indeed, when the E-myc transgenic mice were crossed with an INK4A (p16) knockout mouse, no reduction in apoptosis or acceleration of tumor development was observed. As expected, when p14ARF knockout mice were crossed with the E-myc mice, lymphomas demonstrated significantly reduced apoptosis and rapid tumor onset. Loss of p14ARF in this case appeared equivalent to loss of p53 or overexpression of BCL2. It was convincingly demonstrated that instead of dying by apoptosis, the tumor cells from the p14ARF knockouts entered premature senescence. Thus, in these mice, loss of apoptosis simply revealed an equally sensitive form of cell death that took longer to manifest-premature senescence. This concept may explain in part the poor correlation between apoptotic sensitivity and treatment response that is found in the literature.\[^{[1-3,8,12-18]}\]

The example shown above also illustrates the importance of the dose-response relationship between apoptosis and the treatment agent. Modifying the dose-response relationship for a particular form of cell death will only affect overall treatment sensitivity if this is the most sensitive form of cell death [Figure 1].\[^{[2,3]}\]

**Laboratory evaluation**

One of the principle reasons for the widely held view that apoptosis plays an important role in treatment sensitivity arises from the use of in vitro and in vivo assays that are biased or inappropriate for assessing overall treatment sensitivity.

**In vitro**

A number of in vitro assays are used to assess treatment sensitivity, including those based primarily on the cell growth. The MTT assay, which measures the activity of a mitochondrial enzyme, or assays based on cellular protein or DNA content are often considered as measures of viability and surrogates for treatment sensitivity. However, these assays are chiefly based on measurements of cell number. These assays are normally executed several days after exposure of cells to a damaging agent and as much are influenced not only by cell death but also by transient changes in the rate of cell growth. As many of the same genes that influence cell death also influence cell proliferation, especially in the case of apoptosis, it is difficult or impossible to interpret overall treatment sensitivity from these types of assays.\[^{[2,3,17,18]}\]

As an alternative, several in vitro assays are based on the detection of specific modes of cell death including apoptosis. The least effective assays do not evaluate treatment at the cellular level but instead measure an average or population response. These assays include those that measure changes in the average level of caspase activity or the amount of DNA fragmentation. These assays are useful to demonstrate that apoptosis is occurring but are relatively non-quantitative.

There are two fundamental limitations of all these death-based assays that limit their usefulness as measures of treatment sensitivity. The first is that they give a picture of the response only at the point in time-the time at which the cell population is evaluated. Given the fact that cells in a population can die by several different processes that each operate with different kinetics that
may further vary in different cell types, one can never be sure that the cells that remain viable at the time of assessment will not subsequently die by some form of cell death.\[2,3,11,14\]

Furthermore, as the end stages of apoptosis can result in complete cell destruction, the apoptotic cells are also eliminated from assessment as time progresses. This is particularly a problem when attempting to assess cell death by these assays in vivo. The second major problem is that it is difficult or perhaps even impossible to simultaneously assess all possible modes of cell death. Consequently, these death-based assays can never give a full picture of treatment response.\[2,3\]

The solution to the problem of identifying all forms of cell death was solved in 1956 by Puck and Markus, who developed an assay based on the ability of a single cell to grow into a colony. This “clonogenic assay” has formed the basis of in vitro cellular response studies in tumors and also some normal tissues.\[1-3\] The clonogenic assay tests for the ability of a cell to recover from treatment in such a way that it can proliferate again and form a clone of substantial size (normally evaluated 10-20 days after treatment). It thus can measure the ability of a cell to survive from all possible (known and unknown) forms of cell death. This assay is analogous to the well-accepted and well-proven assays of treatment sensitivity in other organisms such as yeast and bacteria.

The relationship between treatment dose and clonogenic survival established in vitro has successfully predicted the doses required to cure transplanted tumors in vivo even when rates of apoptosis do not correlate.\[2,3,18\]

The modest levels of cell death are assessed using treatment doses that will inactivate several logs of cell kill if assessed by the clonogenic assay. The clonogenic assay is not without problems. The assay often involves plating of dilute concentrations of cells under conditions that are significantly different from those found in vivo. Furthermore, the in vitro conditions ignore the unique micro environmental parameters of a tumor that can be important contributors to treatment sensitivity such as oxygen and cell-to-cell contact.

Several investigators have also pointed out that the long-term culture of cells in vitro can result in selection of resistant clones that are not reflective of the original cells in vivo. In some instances, it is thus desirable to use primary cells derived from tumors in vivo. In this case, it is important that these primary cells can also tolerate in vitro culture conditions without substantial death even without treatment. This can be assessed by the plating efficiency—a measure of the number of cells that retain clonogenic capacity in the absence of treatment. When this value is very low (less than 5%), the predictive power of the clonogenic assay comes into question. Thus, the clonogenic assay is far and away the best in vitro tool for assessing treatment response.

In vivo

There is clearly a need to evaluate cellular treatment sensitivity within the context of the normal environment, a so-called in vivo assay. However, even in this context, several common assays can be highly biased or inappropriate for evaluating treatment response. In particular, assays that are based primarily on evaluating or comparing tumor size (or presence) at fixed times after treatment are difficult to interpret and heavily biased toward tumors that display rapid forms of cell death like apoptosis.\[2,3\]

For example, investigators may treat either spontaneous or transplanted tumors and then evaluate the size of the tumor at one or more times after treatment. This type of experiment is heavily influenced by the intrinsic tumor growth rate, which itself is influenced by cell loss that occurs through death mechanisms such as apoptosis. A tumor that suffers a high loss factor will grow much more slowly than a similar tumor with a lower cell loss factor. To illustrate this problem, consider two different tumors that behave identically with the exception that one has a much slower growth rate because of a higher rate of spontaneous apoptosis [Figure 2]. If it treat each of these two tumors with a dose that produces the same amount of overall cell kill (the same level of survival), it will take substantially longer for the slow-growing tumor to reappear. This may cause misinterpretation of the treatment sensitivity as the “time to relapse” was significantly longer in the slow-growing (apoptotically sensitive) tumor, even though treatment sensitivity and curability are identical.\[2,3,16,17\]

Misinterpretations of treatment sensitivity can also
result from differences in the kinetics of cell death following treatment. A much better way to evaluate the treatment sensitivity of tumors is to measure the growth delay that results from treatment. The growth delay is a measure of the difference in times for treated and untreated tumors to reach a certain size (e.g., three times the starting volume). At this time, the tumor should be growing at a rate that is equivalent again to the untreated tumor [Figure 2]. [2,3] The difference in time required for the control and treated tumors to reach equivalent sizes directly reflects the percentage of cells that survived the treatment. As seen in Figure 2, this value is completely independent of the rate of tumor regression after treatment. To remove the problems associated with comparing tumors that have different intrinsic growth rates, one must use the specific growth delay. [2,3,18] This is simply equal to the growth delay expressed as a fraction of the growth rate of the untreated tumor.

The gold standard of all assays of treatment sensitivity is thus those that evaluates tumor cure. This is evaluated by the so-called TCD50 assay, in which the dose required to cure 50% of the tumors is calculated. In these experiments, cure typically follows a sigmoidal function of treatment dose. Although this is the best of all assays, there has been a steady decrease in its use due to the fact that a large number of mice must be used to accurately define the dose-response relationship. [1-3]

The TCD50 assay also allows one to consider the possible existence of tumor stem cells. This is an old concept that has received renewed interest and implies that only a small fraction of tumor cells are truly clonogenic and capable of unlimited proliferation. Obviously in such a case, it becomes important to evaluate the response of the tumor stem cells and not the response of the bulk of the tumor. As the TCD50 is influenced only by those cells that have the ability to both survive and reform the tumor, it accurately reflects the treatment sensitivity of the relevant cells in the tumor. The behavior of the non-stem cells in this case is irrelevant. [2,3]

**Conclusion**

Our understanding of the molecular basis and regulation of the apoptotic pathway and its importance in cancer has, somewhat paradoxically, clouded our ability to understand the important molecular determinants of treatment response. Experiments demonstrating that apoptosis could indeed influence treatment sensitivity have contrasted with other studies showing that dramatic changes in apoptosis can occur without affecting the overall treatment response. Thus, the question of whether apoptosis, or the genes controlling it, is important for cancer therapy remains controversial.

We have argued that conflicting conclusions over the importance of apoptosis result both from studies with model systems that cannot be extrapolated to most human tumors and from the use of assays that may be heavily biased toward detection of specific modes of cell death. However, in real human cancers, apoptotic sensitivity is dramatically lower and correspondingly also less important (or even unimportant) for treatment response. Proper evaluation of treatment response thus requires careful analysis of each of these forms of cell death prior to making any conclusions. This is most accurately assessed in vitro by use of the clonogenic assay that is capable of integrating all forms of cell death. Similarly, in vivo assays of tumor response must also be conducted in ways that allow evaluation of all forms of cell death.
death. Short-term assays that are influenced by tumor growth rates or the rates of regression should be avoided. Proper selection and interpretation of these assays is necessary for future experiments aimed at evaluating the importance of apoptosis and other determinants of cell death and survival.

Acknowledgement

I sincerely acknowledge to Andrew H. Wyllie, Kerr JRF, Currie AR, Bradly G. Wouters, Roland K. Chiu, David A. Gewirtz, Shawn E. Holt and Steven Grant for their Help and Support as well as for preparing this article.

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Cite this article as: Tiwari M. Apoptosis and survival. Indian J Hum Genet 2011;17:120-5.
Source of Support: Nil, Conflict of Interest: Nil