Counting Heads in the War against Cancer: Defining the Role of Annexin A5 Imaging in Cancer Treatment and Surveillance

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Abstract
The unveiling of the heterogeneous nature of cell death modes has compromised the long-lived consensus that cancer treatment typically kills cancer cells through apoptosis. Moreover, it implies that measures of apoptosis may be misleading indicators of treatment efficacy. Simultaneously, it has become clear that phosphatidylserine exposition, traditionally considered a hallmark of apoptosis, is also associated with most other cell death programs, rendering phosphatidylserine an attractive target for overall cell death imaging. Annexin A5 binds with strong affinity to phosphatidylserine and hence offers an interesting opportunity for visualization of aggregate cell death, thus providing a fit benchmark for in vivo monitoring of anticancer treatment. This might be of significant value for pharmacologic therapy development as well as clinical monitoring of treatment success. (Cancer Res 2006; 66(3): 1255-60)

Introduction
Whereas cancer proceeds to approach the world’s leading cause of death position, oncologic medicine is adapting its strategy regarding cancer treatment, focusing to a higher extent at individual differences in drug responses. The underlying philosophy of this personalized medicine approach is to identify what drug works best in which patient at what time, to be able to provide personally tailored therapy and hence improve success of treatment. Because this strategy requires fast information on whether or not a drug is working in individual patients, demand for more rapid and reliable readouts of treatment efficacy is soaring. Besides improving and speeding up the development of novel therapies, personalized treatment could benefit patients directly, by identifying the optimal therapeutic agent while simultaneously decreasing side effects suffered from ill-working treatments.

To measure treatment efficacy in vivo more rapidly than with conventional techniques, such as computed tomography or positron emission tomography imaging with glucose analogue 18F-fluorodeoxyglucose, other molecular imaging approaches (1) than the latter have received growing attention. Cornerstone of these novel approaches is the finding that the degree of cytoreduction in response to cancer treatment directly correlates to both overall disease response to treatment and rate of survival (2). Although this implies cell death quantification to represent a reliable benchmark for therapy evaluation, the revealed diversity in cell death mechanisms renders its practical feasibility less straightforward than the theory suggests. Recent findings strongly indicate that the vast majority of cell death mechanisms can be detected and visualized in vivo with Annexin A5, which senses and binds to lipid alterations on the membrane surface of dying cells, irrespective of their dying mechanism (and thus does not just recognize apoptosis, as has long been believed). Therefore, cell death imaging with Annexin A5 might circumvent the death mode diversity problem. This review aims to provide an outline of the developments and arguments that have led to this conclusion.

Basic Model of Cancer Development
The unraveling of the complex process of cancer development has been a key clinical target throughout the last decades. Vast amounts of research have crystallized into a hypothetical basal model of tumorigenesis, in which the impairment of cells’ natural defense against mutations is recognized as a crucial initiator step (3). This situation is called “genomic instability” and arises when mutations occur in genes responsible for either DNA maintenance (caretakers), such as repair genes, or cell cycle government (gatekeepers). Gatekeepers represent the organism’s ultimate safeguard of tissue homeostasis, due to their ability to induce cell cycle arrest or apoptosis in cells following a range of stresses, including DNA damage (4). A typical gatekeeper example is the p53 tumor suppressor protein, which can initiate programmed cell death upon detection of genomic stress (5, 6), hence removing the potentially noxious cell from its environment. Its essential importance for preventing cancer development is reflected by the fact that more than half of all human tumors reveal mutations in the p53 gene (7). Upon disabling of these caretaker and gatekeeper controls, cells become susceptible to the accumulation of numerous DNA mutations without being repaired or deleted through apoptosis. In contrast to normal cells, these genomically unstable cells are capable of acquiring sufficient mutations to develop the key features of malignant cells (8).

Apoptosis in Action: the Cancer Treatment Paradox
It is generally accepted that gaining “immunity” to apoptosis represents an essential step in cancer development. Strangely, this notion has long coexisted with the prevailing idea that both chemotherapy and radiotherapy kill tumor cells indirectly, by means of damaging DNA and subsequently relying on the cell’s internal apoptotic cascades to finish the job. In fact, numerous studies have used measures of apoptosis to evaluate cancer treatment efficacy (9, 10). Moreover, these studies were usually capable of correlating drug-induced apoptotic activity with overall levels of cell death as confirmed with histology as well as with clinical responses (11).
However, under the assumption that cancer cells are resistant to apoptosis following DNA damage, a logical inference would be that normal tissues should be more vulnerable to cancer therapy than tumor cells. In practice, this seems not to be the case. When using in vitro clonogenic survival assays to measure overall cell death response to radiotherapy in a variety of normal and malignant murine tissues, no systematic differences between the two groups are observed (12). Moreover, tumors with evident defects in the apoptotic pathway should be expected to be more resistant to these therapies than tumors with intact pathways. After evaluating extensively the huge arsenal of available experimental and clinical data, Brown and Attardi recently concluded that at least for nonhematologic cancers, no such pattern could be distinguished (13). The discussed data comprise a spread of apoptotic deficiencies, including p53 knockouts (14, 15) and models of overexpression of the proapoptotic protein Bcl-2 (16), as well as other members of the Bcl-2 family (17). Together, these findings strongly indicate that the apoptotic capabilities of cells have marginal influence on cell survival following DNA damage. This implies that the consensus of cancer therapy typically and specifically triggering apoptosis cannot and should not be maintained.

How then does therapy kill tumor cells? And how can the described data be synthesized with the occasional success of apoptosis measuring therapy evaluation assays? To illuminate this paradox, we first need to take a closer look at the phenomenon of cell death.

The Different Faces of Cell Death

The classic dual division of cell death, as originated in the seventies (18), distinguished between necrosis and apoptosis (programmed cell death or PCD). This model recognized necrosis as the common insult-induced type of cell death, characterized by cell swelling, membrane rupture, and subsequent cytoplasmatic content release in the cell’s direct environment, hence invoking undesirable inflammation. Its stereotypical counterpart, apoptosis, was perceived to embody a highly organized mode of cellular “suicide,” with a group of biological executioner proteases called caspases responsible for its morphologic and biological features (19). The most obvious are cytoplasmatic shrinkage, chromatin condensation, DNA degradation, membrane blebbing, subsequent formation of apoptotic bodies, and importantly, no inflammatory response. This dual division, however, has been proven too simplistic to maintain.

Rather than a sharp boundary, research has unveiled considerable overlap between necrosis and apoptosis. For instance, cells undergoing apoptosis (which is an energy-consuming process) have shown the ability to switch to necrosis upon energy depletion (20, 21), whereas the opposite may also occur when the noxious stimulus driving a cell into necrosis is removed before the end (22). Interestingly, it was also shown that PCD-inducing signaling from the cell’s environment may result in both apoptotic and necrotic phenotypes of cell death, depending on cell type or cellular content (23). This not only implies that both modes might be closely intertwined, but moreover, that necrosis can be the result of a regulated initiation of cell death, in contrast to the widely perceived notion of necrosis as a passive process following damage.

In 2001, Leist and Jäättela pointed out that caspases, although renown as the central executioners of apoptosis, are not always involved in PCD mechanisms (24). They suggested a less rigid model, in which cell death is regarded a hybrid event on the gliding scale between two extremes (i.e., apoptosis and necrosis; ref. 24). The precise cell death phenotype is determined by the resultant of cell type, stimulus, and competition of different PCD mechanisms. This model is not without its merits in a sense of providing a new paradigm for studying cell death and its modes of appearance.

Currently, several specific types of cell death have been identified in tumors as a result of DNA-damaging agents, as has been reviewed in more detail by Brown and Attardi (13) and Okada and Mak (25). In addition to classic apoptosis, cancer cells may also die from necrosis, mitotic catastrophe, autophagy, or be functionally deleted through what is called senescence (13). Concisely, mitotic catastrophe can occur during attempted mitosis following unsuccessful chromosome segregation, a situation that requires malfunctioning of multiple cell cycle checkpoints (26). Catastrophic cells are typically large and nonviable, containing multiple nuclei with chromosome fragments. Their compromised nuclear blueprint eventually causes cell death, which may or may not occur through apoptosis, yet if so, it usually does so in a p53-independent manner (27). Autophagy represents genetically regulated self-digestion of the cell. In a caspase- and p53-independent fashion, organelles are incorporated in autophagosomes, subsequently merging with lysosomes to cause content degradation (28). In contrast, senescent cells do not necessarily die, but irreversibly stop dividing, long before their telomere-restricted maximum amount of divisions has been completed (25), resulting in functional deletion. Consequently, it has been argued that despite of its crucial role in tumorigenesis, apoptosis might play a relatively modest role in cancer response to treatment, because its pathways are assumed to have been compromised. More likely, mitotic catastrophe and senescence are responsible for the major share of treatment efficacy (29).

Concluding, dependent on cell and environmental context, cell death may present in many different forms, rendering the dual necrosis/apoptosis model of cell death a shortcoming framework from which to study and evaluate cell killing by antitumor agents.

Evaluating Tumor Treatment Efficacy

The extended model of cell death sheds new light onto the paradoxical findings regarding apoptosis as described earlier. To start with, the hybrid nature of cell death makes it conceivable that studies (mistakenly) focusing on apoptosis as a measure of overall anticancer treatment efficacy will indeed find apoptotic characteristics, in some cases, even quantitatively related to tumor regression. More importantly, however, the model provides theoretical backing for the poor correlation between tumor sensitivity to treatment and the condition of apoptotic pathways, because the cell has multiple alternative pathways at its disposal when apoptosis fails.

Unfortunately, the corollary of the limited role of apoptosis in tumor treatment response is that measures of apoptosis do not necessarily reflect overall cell killing. This could easily lead to wrongful conclusions in cases where tumor response is primarily effected through mitotic catastrophe or autophagy. This problem was recently elaborated by Brown and Attardi (13). They pointed out that the use of assays measuring clonogenic potential of tumor cells after treatment in vitro (or, in short, clonogenic survival assays) leads to much more reliable efficacy evaluation. This is because this method considers the net product of all types of cell death as well as growth arrest over a longer time span. In fact,
it has been shown that when comparing treatment responses of tumor cells with either intact or compromised apoptotic pathways, assays focusing on the degree of induced apoptosis report lower sensitivity of the compromised cells, whereas clonogenic survival assays reveal no difference in long-term net effectiveness between the two (13). These results unveil the significance of alternative death mechanisms, stressing the need for caution while interpreting the conclusions of numerous past studies.

The vital shortcoming of clonogenic assessment, however, is that irrespective of its reliability, it is not feasible for in vivo imaging. For that reason, it cannot incorporate the complex interactions of the cellular environment and more practically, it does not yield a solution for the clinical need for rapid therapy evaluation of patient treatment. In contrast, new insights concerning the membrane association of phagocyte attractant phosphatidylserine on dying cells provide an attractive opportunity for noninvasive in vivo imaging of cell death following antitumor treatment. Moreover, evidence indicates that this approach might very well circumvent the above described cell death type–related problems.

**Annexin A5: Detection of the Cell Death Signature**

Annexin A5 is an endogenous protein that binds with high affinity and specificity to phosphatidylserine, which is presented on the cell membranes of apoptotic cells (30–33). As this quality is hardly affected by labeling with optical or nuclear probes, in vivo molecular imaging of cell death using Annexin A5 (Fig. 1) has been applied successfully in a variety of (patho)physiologic conditions, including cardiovascular and oncologic medicine (11, 34–42).

It has long been assumed that only apoptotic cells externalize phosphatidylserine. Accumulating experimental data, however, indicate that phosphatidylserine externalization during cell death is by no means an exclusive apoptotic event. The first step in this respect was the finding that activation of caspases is not a necessary condition for phosphatidylserine externalization and that the latter occurs in nonapoptotic cell death phenotypes just as well (43–46). Moreover, it has recently been shown by several groups that even necrotic cell death is associated with active presentation of phosphatidylserine at the cell membrane (47). For both apoptosis and necrosis, Brouckaert et al. showed that efficiency of phagocytosis is markedly compromised by competitive occupation of phosphatidylserine with recombinant Annexin A5, hence reaffirming the functional importance of phosphatidylserine in both cell death modes (26, 48, 49). Additional studies have revealed that regarding phosphatidylserine externalization, mitotic catastrophe, and autophagy form no exception (48). Senescent cells, logically, do not express phagocyte attractants, because they do not necessarily die. Nonetheless, their eventual perishing typically

![Figure 1. Schematic presentation of the principle underlying cell death imaging with labeled Annexin A5. Living cells actively internalize phosphatidylserine (PS, red dots) to the inner leaflet of their cell membrane. Upon activation of cell death mechanisms, the opposite occurs: phosphatidylserine becomes actively externalized and is presented at the membrane surface to attract phagocytes. Annexin A5 binds with high affinity to externalized phosphatidylserine and can be labeled with molecular tracer groups to allow for in vivo cell death monitoring with a variety of imaging modalities.](image-url)
occurs through either apoptosis or mitotic catastrophe, in both cases accompanied by phosphatidylserine externalization (10, 50).

It needs to be stressed that only when used with a second marker showing an intact cell membrane can Annexin A5 be assumed to specifically monitor externalization of phosphatidylserine. Under controlled laboratory conditions, these conditions can be met. However, when used as a single agent in patients, interpretation of Annexin A5 is complicated by the ability of the protein to bind both externalized as well as internal phosphatidylserine when the plasma membrane is leaky, as occurs in necrosis. However, because the latter cause of membrane disruption is just as well associated with dying of cells, it can be argued that this would not necessarily compromise the final imaging objective, which is measurement of overall cell death.

These novel insights obviously yield important implications towards the Annexin A5 imaging protocol. Whereas the bad news is that Annexin A5–positive results in a variety of studies from the past might have mistakenly been attributed to apoptosis alone, the good news concerning in vivo cancer treatment evaluation is exciting. After all, if phosphatidylserine exposure is a hallmark of the dying cell, irrespective of the involved cell death program, then phosphatidylserine detection using Annexin A5 overcomes the death mode-related problems as described in earlier sections. This transforms Annexin A5 into a very promising candidate to meet the need for rapid in vivo imaging of aggregate cell death responses to tumor treatment.

**Annexin A5 Imaging of Treatment Efficacy: Caveats and Opportunities**

Important to remind regarding imaging of cancer therapy efficacy is the fact that aggressive tumors are notorious for displaying much higher levels of cell turnover than benign processes. This implies that snapshot imaging of Annexin A5 binding might be an inadequate measure of tumor response to treatment, prone to mistaking tumor aggressiveness for treatment success. Thus, it seems more advisable to base therapy efficacy evaluation upon Annexin A5 uptake compared with baseline images, preferably measuring signal uptake at multiple time points. Additionally, imaging over too short a time period following therapy could entail a pitfall, because cell death programs other than apoptosis might not become discernible at equally short time horizons. Further research will be required to identify optimal imaging timeframes, which are likely to depend on cancer type and treatment.

Taking the above into account, the use of Annexin A5 imaging for monitoring cancer treatment offers attractive perspectives in both the pharmaceutical and clinical arena. Regarding the

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**Figure 2.** Example of in vivo evaluation of anticancer drug response with fluorescence molecular tomography using Annexin A5/Cy5.5 in mice. Lewis lung carcinoma cells, sensitive and resistant to cyclophosphamide, were injected into the right and left mammary pads, respectively. A to D, four consecutive tomographic slices with (D) positioned closest to the ventral surface, showing higher tumor response to treatment in sensitive tumor cells. Images reproduced with permission from Vasilis Ntziachristos, Ph.D. (Center for Molecular Imaging Research, Massachusetts General Hospital and Harvard Medical School, Boston). Copyright 2004, National Academy of Sciences.
pharmaceutical industry, fast and reliable in vivo evaluation of aggregate cell death could accelerate the search for and development of novel antitumor strategies and compounds. Herein, the combination of Annexin A5 with rapidly improving imaging techniques for small animal (Fig. 2) and clinical imaging can be expected to yield significant benefits (11, 51). Moreover, the current attention shift towards personalized medicine and local drug delivery through targeted therapy will likely increase the need for live molecular approaches of cell death imaging even further in the future.

Concerning the evaluation of cancer therapy based on Annexin A5 uptake in patients, initial results have been very promising (Fig. 3), although significant additional clinical research is still required. In addition, further improvement of the clearance and biodistribution profile of the radioligand is also recommended and feasible. 99mTc/Annexin A5 has been used successfully in clinical studies measuring cell death in lesions above the diaphragm. The major drawback of this radioligand seems to be its renal retention, which makes it difficult to measure cell death in the abdominal region. Replacing the HYNIC group with an endogenous chelation site for 99mTc decreased renal retention to levels allowing measurement of cell death in the abdomen (ref. 52). The potential gains from this therapy evaluation strategy involve aspects of time, cost, and side effects. As cancer treatment is currently primarily evaluated through awaiting macroscopic regression, often taking weeks, patients bear the risk to suffer from possibly ineffective therapy. Thus, faster recognition of treatment (in)efficacy should allow for much faster switching to more successful treatment alternatives, significant reduction of unnecessary side effects, and consequently marked cost reduction.

**Outlook**

Molecular imaging of apoptosis with Annexin A5 seems to be a fruitful approach for cell death recognition in vivo. Novel insights reveal that Annexin A5 cell death recognition is not limited to apoptosis but extends to the vast majority of alternative cell death mechanisms. Thus, it meets the growing oncologic demand for a general cell death marker, which has arisen from the discovery that a wide spread of cell death mechanisms is involved in tumor response to treatment. In the future, the Annexin A5 imaging protocol could contribute substantially to the trend of personalizing cancer medicine by providing a rapid readout of treatment efficacy and hence be of value for both research and development and clinical monitoring of patient treatment.

**Acknowledgments**

Received 8/24/2005; revised 11/30/2005; accepted 12/15/2005.

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Figure 3. Evaluation of the anticancer therapy efficacy in lymphoma patients with single photon emission computed tomography nuclear imaging using 99mTc/Annexin A5. A-D and E-H, total body (nontomographic), transverse, sagittal, and coronal images of a patient before and after treatment, respectively. Images obtained after radiotherapy show clear uptake of 99mTc/Annexin A5, revealing positive cell death response to treatment. Images reproduced with permission from Rick Haas, M.D. (Department of Radiotherapy, The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands). Copyright 2004, Int. J. Radiation Oncology Biol. Phys., Elsevier Publishing.


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