What Do Shifts in Indicators of Apoptosis Indicate about the Cancer Process?¹⁻³
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Expanded Abstract

Overview
Apoptosis is genetically programmed cell death (1). It is defined by specific morphological criteria whose hallmark is condensation and fragmentation of the cell nucleus with endonuclease cleavage of DNA, but it is mediated by more than 1 biochemical pathway. The 2 major pathways of apoptotic cell death are the intrinsic or mitochondrial pathway and the extrinsic pathway, which is dependent on death receptor–ligand interactions. These pathways depend on execution caspases to initiate the DNA cleavage and catabolism of the cytoskeleton that results in cell death. There also is a programmed cell death (PCD)⁴ pathway that is independent of caspases, and, in addition to these PCD pathways, cancer cells can die through necrosis, mitotic catastrophe, autophagy, and senescence (2,3). Necrosis, which is mediated by processes outside the cell, is unlike apoptosis or PCD because inflammation can result from release of the cell contents. Mitotic catastrophe likely occurs during mitosis and is thought to be secondary to missegregation of chromosomes and/or cell fusion. It can lead to apoptosis. Autophagy is a caspase-independent form of cell death in which cells digest themselves. Senescence occurs when metabolically active cells no longer divide and may increase in size.

The intrinsic or mitochondrial pathway of PCD is regulated by a family of proteins called the BCL-2 family. The first member of this family to be identified was an antiapoptotic protein named BCL-2 (1). The name was given because it was cloned from a B-cell lymphoma as a chromosomal translocation is which the coding sequence of BCL-2 is placed under transcriptional control of the Ig heavy-chain locus, resulting in significantly increased levels of BCL-2 expression and resistance to apoptosis. Subsequently, it was determined that the human BCL-2 gene was the mammalian counterpart of a gene in the nematode Caenorhabditis elegans that regulated apoptotic cell death. The gene cascade that ultimately governs the intrinsic apoptotic process (that BCL-2 is a member of) was defined in genetic studies performed using the C. elegans developmental model.

Unlike C. elegans, in which there is only 1 BCL-2 functional homolog, mammalian species express multiple family members that include both antiapoptotic (such as BCL-2) and proapoptotic members. The relative concentrations of the antiapoptotic and proapoptotic BCL-2 family members act as a rheostat to determine the propensity of the cell to undergo apoptosis by regulating activation of caspases through changes in mitochondrial membrane permeability and release of cytochrome c. The caspases are the executioners of apoptosis and are synthesized as inactive procaspases and subsequently activated by cleavage. Like the BCL-2 family, caspases also represent a family of related proteins with some overlapping functions. Initiators of apoptosis (IAPs) are another family of proteins involved in the regulation of apoptosis. They inhibit apoptosis by blocking caspase action. The extrinsic or cell death receptor pathway works independently of the BCL-2 family (3,4). It is triggered by death ligands including tissue necrosis factor-α (TNF-α), Fas ligand (FasL), or TNF-related apoptosis-inducing ligand (TRAIL). After death-induced activation, the receptors can interact with adapter proteins such as Fas-associated via death domain (FADD), which are homologous to proteins that act in the intrinsic pathway (Apaf-1). Similar to the extrinsic pathway, the extrinsic pathway eventually activates caspase proteins as the “executioners” of apoptosis, p53 is a defined tumor suppressor gene whose loss of function through either mutation or functional inactivation in cancer processes can suppress the apoptotic response triggered by DNA damage and other stimuli (5).

Senescence is a state of permanent cell-cycle arrest. It is thought of as a normal physiological process that acts to inhibit the development of cancer by limiting the number of replicative cell cycles that a somatic cell can undergo (6). Most frequently it

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⁴ Abbreviations used: FADD, Fas-associated via death domain; IAP, inhibitors of apoptosis; PCD, programmed cell death; TNF-α, tissue necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling.
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results from progressive telomere shortening in somatic cells as a result of the absence of telomerase, a specialized replicase that is responsible for maintaining the telomeres at the ends of chromosomes. If a cancer is to develop, the initial transformed cell must prevent telomere shortening to maintain an unlimited replicative potential. Increased expression levels of telomerase are linked to development of cancer. There are other stresses that can induce senescence without telomere shortening, such as DNA damage and p53 activation. At the same time, telomere shortening by itself is linked to activation of p53. Induction of senescence is a genetic program. In mice, when the genes that regulate entry into senescence [p53, CDKN1A (p21), CDKN2A (p16), Rb] are inactivated and entry into senescence is impaired, cancer incidence is increased.

**Discussion**

Defects in apoptosis and in the induction of nonapoptotic cell death contribute to the development of cancer (3,6). On the other side, induction of cell death, through apoptosis or other mechanisms, is a hallmark of successful cancer treatment. Significantly, induction of apoptosis does not appear to be the most potent means of inducing cell death by cancer chemotherapy and is not frequently a good biomarker of treatment response (7). At the same time, overexpression of cell survival genes or decreased expression of cell death genes does correlate with prognosis in some cancers. Numerous pharmacological agents are in the developmental pipeline in the hopes of improving response rates for multiple different types of cancer (8–10).

Cells that are undergoing apoptotic cell death can be recognized in tissue after routine processing with hematoxylin and eosin (H&E) staining, but the technique requires an experienced observer to execute successfully. Similarly, electron microscopy can be used, but this requires special equipment and is both expensive and laborious. Because the endonucleases active during apoptosis cleave the DNA at discrete ~180-base pair intervals dictated by the nucleosome structures, techniques that capitalize on recognizing this cleaved DNA were developed. DNA can be isolated and fractionated through a gel, and if a significant number of cells are undergoing apoptosis, the DNA will appear as a ladder. The sensitivity of the technique can be improved by isotope or other labeling of the DNA. TDT-mediated dUTP nick-end labeling (where TDT is a terminal deoxynucleotide transferase enzyme) or TUNEL assay can be used to label the fragmented DNA ends in tissue in situ and combined with either immunohistochemistry or immunofluorescence to localise the cells (11). A third approach relies on identification of the activation of specific enzymes during apoptosis (12). Activation of both cysteine-dependent aspartate-specific proteases (caspase)-3 (and/or other caspases), and poly ADP-ribose polymerase (PARP) can be detected by either in situ immunohistochemistry or immunofluorescent techniques or by Western blotting of protein extracts from tissue.

In tissue culture cells DAPI staining can be used to enhance visualization of fragmented nuclei, and annexin V staining can be used to detect the increase in externalized phosphatidylserine residues as the cell membranes are disrupted during apoptosis, but these techniques are less useful for recognition of apoptotic cells in intact tissue. Similarly, the commercially available ELISA kits that detect activated caspases have proven more reliable in tissue culture cell studies than in intact tissue investigations. Changes in expression of apoptotic pathway genes are a fourth approach that has been used successfully for detection of apoptosis in intact tissue. Immunohistochemical or immunofluorescent techniques can be used to monitor changes in protein expression in situ, or Western blotting of protein extracts or measurement of steady state RNA expression levels for BCL-2 family genes, Fas, Fasl, TRAIL and related genes, and caspase family genes. Cleavage of cytokeratin 18 by activated caspases can be detected using a monoclonal antibody, but its sensitivity in tissue remains under investigation.

The most useful techniques supply both detection and quantification of apoptosis because it is not the presence or absence of apoptosis that must be determined in studies of cancer progression but rather the change in the relative amounts of apoptosis. In this regard, the most valuable measures in tissue are those that can be used to calculate an apoptotic index. The apoptotic index is the percentage of a specific type of cell that is undergoing apoptosis. Rates of apoptosis and proliferation can be compared to determine whether the net effect of a specific intervention results in a net loss of a target cell population. The in situ–based techniques (e.g., TUNEL, caspase-3 detection) are the most useful to derive an apoptotic index. Changes in expression of specific apoptotic pathway genes are an important adjunct to the in situ–based techniques. It is well known that cells that die through necrosis can on occasion be detected in a TUNEL assay, and therefore it is critical that accurate measurements of apoptosis include the application of more than one technique. The combination of morphological analysis, an in situ–based technique to detect either DNA fragmentation or caspase activation, and apoptotic pathway gene expression assays is the best approach.

**Research needs**

A current challenge in detecting apoptosis is to try to make the detection and quantification process less time consuming and laborious. ELISA-based assays or other approaches that could be performed on small quantities of tissue extracts would be preferable, but at present these do not appear to be reliable enough. It is difficult to calculate an accurate apoptotic index on small tissue samples. One needs a minimum of several hundred cells (optimally 500–1000 cells) for an accurate determination. There is considerable incentive for commercial enterprises to develop accurate reproducible assays that can be performed on small tissue samples, but it is a challenging problem. Development of serum markers that can detect changes in tissue apoptosis is another compelling research area that has shown modest progress. Measurement of cytochrome c (13) and detection of cleaved cytochrome 18 (14) are 2 approaches that have been used. Because cells can die from nonapoptotic mechanisms as well (15), methodologies to detect these mechanisms are equally important.

Necrosis can be identified by alterations in permeability to trypan blue or other vital dyes. Mitotic catastrophe is defined by the presence of micronuclei after mitosis, and multinucleated cells are readily identifiable on light microscopy. Senescent cells can be identified by the presence of senescence-associated β-galactosidase. Autophagy is defined by the identification of cyttoplasmic vacuoles using monodansylcadaverine combined with the lack of margined nuclear chromatin. These assays can be made quantifiable by counting the number of cells demonstrating the features that define the process in the total cell population and determining a percentage. The most useful techniques supply both detection and quantification of apoptosis because it is not the presence or absence of apoptosis that must be determined in studies of cancer progression, but rather the change
in the relative amounts of apoptosis. In this regard, the most valuable measures in tissue are those that can be used to calculate an apoptotic index. The apoptotic index is the percentage of a specific type of cell that is undergoing apoptosis. Rates of apoptosis and proliferation can be compared to determine whether the net effect of a specific intervention results in a net loss of a target cell population.

The in situ–based techniques (e.g., TUNEL, caspase-3 detection) are the most useful to derive an apoptotic index. Changes in expression of specific apoptotic pathway genes are an important adjunct to the in situ–based techniques. It is well known that cells that die through necrosis can on occasion be detected in a TUNEL assay, and therefore, it is critical that accurate measurements of apoptosis include the application of more than one technique. The combination of morphological analysis, an in situ–based technique, to detect either DNA fragmentation or caspase activation and apoptotic pathway gene expression assays is the best approach. Development of real-time histological methods that can be used to follow apoptosis and tissue remodeling will help speed analysis of tissue specimens. Nondestructive technologies such as reflectance confocal microscopy can be used to optically serial section tissue to follow the remodeling process (Fig. 1) (16). The same tissue can then be used for other biochemically based assays, providing both descriptive histology and investigation of different molecular mechanisms on the same specimen.

In conclusion, development of multiplexed assays that are both more sensitive and less time-consuming than current methodologies will help investigators better understand the role of the different death processes in therapeutic response.

**Figure 1** Correlation between salivary gland images from reflectance confocal microscopy (RCM) and conventional H&E during reversal of dysplasia in an experimental mouse model. (A) The experimental mouse model is a conditional model of salivary gland cancer that proceeds through well-defined stages of reversible and irreversible dysplasia (17,18). When oncogene (Simian virus 40 T antigen or TAG) expression is downregulated by doxycycline (dox) administration at 4 months of age, the dysplasia reverses over a 14-d time period (18). This model was used to compare histological results from reflectance confocal microscopy to conventional H&E staining during tissue remodeling. Optical sectioning is accomplished within 5 min per gland and provides digital records of the findings for further analysis when required. Salivary tissue exposed to 5% acetic acid before imaging showed improved contrast (16). Comparison of submandibular salivary ductal structure in CRIDS mice before (A, C) and 14 days after (B, D) administration of doxycycline to downregulate TAG oncogene expression. Black arrows indicate ductal cells with nuclei that demonstrate high reflectance under RCM and intense blue staining from hematoxylin under light microscopy after H&E staining. White arrows indicate ductal cells that have undergone reversion with redifferentiation and the appearance of normal striations in the cytoplasm. Note that the majority of ducts demonstrate clear reversion. We have selected an area that demonstrates a ductal structure that has not yet completely reverted (black arrows, B) for contrast. (C, D) Z-stacks are a series of optical sections that are saved as digital images. Comparison of Z-stacks before and after doxycycline administration. White boxes outline the optical sections illustrated in A and B at higher power. Note that whereas all the sections through the "No Dox" gland demonstrate cells with high reflectance, the percentage of cells demonstrating this intensity of reflectance are significantly reduced after doxycycline treatment (D).

**Literature Cited**


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