REVIEW ARTICLE

# Apoptosis detection modalities: A brief review

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## Abstract

Apoptosis or programmed cell death is a specific cellular event with distinct morphological, histological, molecular characteristics, and biochemical mechanisms. It plays an important role in normal turnover of the cell, development, and its function. Inadequate apoptosis (either too little or too much) is one of the major causes for various pathologies such as neurodegenerative disorders, ischemic, autoimmune diseases, and various forms of cancer. Since controlled apoptotic programs can produce changes in cell death pattern, the genes, and proteins that regulate apoptosis are potential future drug targets. Hence, detection of apoptotic cells will pave a new path for cancer diagnostics, prognosis, and therapy. The aim of this review article is to discuss various methods of apoptosis detection, from traditional approaches to recent advanced molecular methods.

## Introduction

Oral cancers are one of the main health problems in developing countries like India. According to statistics, in India, cancer related to oral cavity, is most common in men and the third most common in women.[1]Most of the oral cancers are epithelial in origin (squamous cell carcinoma) and are associated with tobacco chewing habit. Early diagnosis of cancer, greatly increases the cure rate, with less impairment and deformity.[2]

The word apoptosis means "dropping off" or "falling off" which is derived from the Greek word, which refers to the petals falling off from flowers or leaves from trees. Kerr FR, in 1972, first coined the term "apoptosis" to describe, "programmed cell death." It is a process of cell death that is involved in the normal cellular development and aging process, which is distinct from necrosis.[3]

Apoptosis is a form of "co-ordinated and cell death programmed internally" having significance in a variety of physiologic and pathologic conditions. It is an active, well regulated cellular process where individual cells are activated to endure self-extinction in a mode that neither injures the neighboring cells nor evoke any inflammatory reaction, unlike necrosis.[3]

Current research has helped us understand apoptosis better. Impairment in the apoptosis mechanism can cause autoimmune diseases, ischemic heart disease, and brain disease apart from cancer. Identifying and understanding apoptosis has become important besides its prognostic significance.[2]

## Various Methods of Apoptosis Detection Are

1. Light microscopy
2. Special stains/fluorescent stains
3. Immunohistochemistry (IHC)
4. Electron microscopy
5. Polymerase chain reaction (PCR)
6. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)
7. DNA agarose gel electrophoresis
8. Flow cytometry (FCM)
9. *In situ*3 - end labeling method (ISEL)

10. Western blotting and caspase colorimetric assay
11. Nuclease assay.

### Light microscopy

Apoptosis usually involves single cells or small clusters of cells. This can be appreciated in tissues stained with routine hematoxylin and eosin stain. Chromatin material get condensed to granular masses that is sharply delineated along the nuclear envelope, cell shrinkage, cellular, and nuclear outlines get convoluted, and nuclear fragmentation may be seen. The apoptotic cell breaks into membrane bound bodies that contain only nuclear remnants.[4]

### Special stains/fluorescent stains

The new apoptotic detection methods include fluorescent dyes such as ethidium bromide- acridine orange (EB/AO), DAPI (4, 6-diamidino-2 phenylindole), propidium iodide (PI), Hoechst staining and Annexin V staining. They bind to DNA in a specific manner and provide a definite analysis of chromatin condensation by fluorescent microscopy. Both live and dead cells can be stained by vital dyes like AO stain. EB stains only those cells that have lost the membrane coherence.[5]

In EO/AO dye:

- Live cells will appear uniformly green due to normal chromatin present in the nuclei.
- Apoptotic cells have condensed/fragmented chromatin in their nuclei. These cells in early stages will stain green with bright green dots in the nuclei due to condensed chromatin and fragmented nuclei. Apoptotic cells in the final stages stain orange after incorporating EB.
- PI stains only cells that have disrupted/loss of membrane integrity.

When cells were double stained with Hoechst stain and PI stain, almost all cells were classified into:

a. Live cells with intact nuclei that is positive for Hoechst stain
b. Necrosed cells with positive nuclei staining with PI
c. Apoptotic early stage cells with fragmented nuclei that is positive for PI stain
d. Apoptotic terminal stage cells with fragmented nuclei that is positive for PI stain.[6,7]

### IHC

The immunohistochemical apoptotic cell detection uses antibodies against substrates like. (1) Caspases 3, (2) M30, (3) Annexin V, and (4) p53.[8]

1. Caspases 3

   Caspase3 is very essential for the apoptosis execution, as it is responsible (partially/totally) for breakage of many key proteins known as proteolytic cleavage. Caspase 3 plays an important role in mediating apoptosis. Active caspase 3 undergoes cleavage which in turn activates other caspases. Neo-epitopes are formed by the enzymatic activation of pro-caspases which are used as antigens to generate specific antibodies. The cleaved final product can be immunodetected.[9]

   Active caspase 3 detection *in situ* is considered direct, specific, sensitive indicator and a reliable method to detect and quantify apoptosis. This is more accurate than older detection techniques based on DNA fragmentation or caspase substrate cleavage. Caspase activity inhibition to treat a variety of apoptosis-related diseases is considered as a new novel therapeutic strategy.[8]

2. M30

   M30 neoantigen is a neoepitope in cytokeratin 18 (CK18), which is available during cleavage of caspase event. M30 is specific to apoptosis, not detected in normal or necrosed cells. It is a monoclonal antibody and a fragment of CK18 cleaved at Asp396 (M30 neoantigen) is specifically recognized. In salivary glands, on trophoblastic tissue both *in vitro* and *in vivo* this marker is validated.M30 can be checked in fresh tissue samples as well as formalin fixed paraffin embedded tissues. M30 can be considered as an accurate apoptosis detection marker.[8,10]

3. Annexin V

   Plasma membrane changes are one of the first characteristic features of apoptosis detected in living cells. Phosphatidylserine (PS) is present on the cytoplasmic face of the plasma membrane. Detection of PS is a very significant feature that is normally used to detect apoptosis. During the apoptotic process, there is translocation of PS to the outer surface of the plasma membrane. Annexin V acts as an extrinsic membrane that detects cell surfaces exposed to PS, both *in vitro* and *in vivo*. This isan early detector that is noted before other apoptotic processes such as loss of plasma membrane integrity with condensed chromatin and DNA breaks. Ongoing apoptosis is best detected by Annexin V. When combined with other methods such as with fluorescent isothiocyanate (FITC), fluorescent microscopy using TUNEL-FITC topographic distribution of DNA can be studied.[11,12]

4. p53

   p53 is a tumor suppressor gene located on chromosome 17pl3. It restricts aberrant and disturbed cell growth which occurs due to damage of the DNA, activation of oncogenes, hypoxic conditions, and the normal cell to cell contact loss. It removes excess, infected or damaged cells by apoptosis, hence called cell cycle regulator. The functions of this gene can be hampered during cancers by various mechanisms which include:

- Prevention of p53 activation
- Mutations within p53 gene
- Mutations of functions associated with p53 mediators.

Mutant p53 expression in the tumor specimens often indicates bad prognosis. It is used to suppress tumor growth, prevent chemoresistance and chemotherapy-induced cell death by inducing apoptosis to tumor cells.[13-16]

### Electron microscopic features

Electron microscopy detects the changes at subcellular level, like chromatin condensation, the formation of cytoplasmic blebs that aggregates peripherally under the nuclear membrane.

During apoptosis, plasma membranes remain intact until the last stages. The space previously occupied by the apoptotic cell will be replaced by the adjacent healthy cells.[4]

Scanning electron microscope (SEM) or transmission electron microscope (TEM) can be used to study apoptosis. TEM detects chromatin condensation and convulsions in and around the nuclear membrane that precedes nuclear fragmentation. The condensation of cytoplasm with the disappearance of the microvilli, blebs on the cell surface, and loss of cell junctions may be noted. Genomic DNA cleavage that breaks into multiple fragments of 180-200 base pairs (bp) is the most characteristic feature. Changes in the surface of the cancer cells such as smoothening, structural loss of microvillus, blebbing/shrinking can be studied by SEM.[17-19]

### PCR

Apoptotic DNA cleavage pattern can be studied using PCR technique. It has better sensitivity than commonly used DNA ladder assay. It is effective in detecting, even in sparse cases that have <1% of apoptotic cells. It is a semi quantitative technique that estimates and compares DNA fragmentation in investigated samples.[20,21]

### TUNEL staining (Terminal deoxynucleotidyl transferase dUTP nick end labeling)

TUNEL is one of the best methods to detect DNA fragmentation that occurs during apoptotic mechanisms. This identifies the nicks present in the DNA of each cell or TdT, the addition (2'-deoxyuridine 5' triphosphate) that are labeled secondarily using a specific marker. DNA damaged cells can also be labeled using TUNEL method. It can be applied to cultured cells, tissues, blood samples, materials that contain few apoptotic cells.[22]

### DNA agarose gel electrophoresis method

Apoptosis is detected by the DNA fragmentation, characterized by endonuclease activation with cleavage of chromatin DNA into multiple internucleosomal fragments of roughly 180bp and its multiples (360, 540, etc.). Gel electrophoresis uses this method for apoptosis detection. This method separates substances based on its molecular weight and rate of movement under the influence of an electric field.

DNA agarose gel electrophoresis detects low molecular weight fragmented DNA in apoptotic cell which is analyzed by numerous gel electrophoresis techniques like.

Conventional gel electrophoresis: This method separates DNA with low molecular weight. Characteristic "ladder" pattern that represents discontinuous DNA fragments will be seen.

Pulsed field gel electrophoresis: This detects high molecular weight DNA fragmentation in the range of kilo to megabases, i.e., 50kb with length up to 10 Mbp in apoptotic cells. When the electric field between the electrode pairs is altered, apoptotic DNA can be separated, as they move in a specific way through agarose gel pores after they get reoriented.

Field inversion gel electrophoresis (FIGE): To achieve a net migration, a constant electric field is periodically inverted with the "forward" direction pulse of the higher field for a longer period (or both). FIGE allows separation of DNA as well as protein mixtures based on size ranges which is not achievable by ordinary electrophoresis. Small samples of DNA can be analyzed, integrity checked by FIGE, unlike conventional gel electrophoresis where only large samples can be analyzed.

Single cell gel electrophoresis comet assay (SCGE): SCGE is a precise method of cell death measurement as it visualizes DNA damage of individual cell. As the DNA that gets degraded resembles the shape of a comet when seen on the electropherograms, they are called as comet assay. The quantity of DNA present in the nucleus or "head," the part of DNA that has shifted away from the nucleus forming the tail, gets embedded in the thin agarose gel layer during the electrophoretic separation forming a comet pattern. This detects pH dependent DNA strand breakage. In alkaline conditions, this assay detects both single- and double-stranded breakage, excision repair site, and alkaline-labile sites. It detects double-strand DNA breakage, mainly in neutral conditions which are considered to be suitable for apoptotic detection. The viability of cell can be assessed as dead or living, and cell death type can be interpreted as apoptosis or necrosis.[23]

Apoptotic cells and viable cells can be distinguished as apoptotic cells display large tails and small heads unlike live cells with a large head and small tail. Necrosed cells present nuclear remnants with minute tails. Hence, this concept has been used in apoptosis studies.[24,25]

### FCM

It is a technique of choice for the appropriate apoptosis quantification, a procedure which separates apoptotic and other non-apoptotic cells by staining DNA material. This multiparametric analysis can count, examine and sort microscopic particles that is suspended in a stream of fluid. An electronic detection apparatus that records the forward scatter and the side scatter allows the study of the physical as well as chemical characteristics of cells, thus distinguishes apoptotic cells from other cells.[26-29]

### ISEL

ISEL technique allows accurate identification of single apoptotic cells at the free ends of the DNA by using radioactive or non-radioactive labeling. Quantification of apoptosis is done by detection of fragmentation of DNA on a cell-to-cell basis that will preserve the topological information even in very low frequencies.[8]

### Western blotting and caspase colorimetric assay

Western blotting (immunoblotting, protein blotting) is a core technique in molecular studies that recognizes specific protein from complex mixture of extracted cells.

The three key factors of Western blotting:

- Gel electrophoresis that separates protein mixtures
- The separated proteins that will be efficiently transferred to a solid support
- The specific target protein detection by matching antibodies appropriately.

The visualization of target protein is seen on the blotting membrane, X-ray film or an imaging system as a band. Expression of Bcl-2 and Bax can be examined and analyzed by western blot analysis. Caspase activity can be detected in the presence or absence of caspase inhibitors by western blot analysis and fluorometric protease activity assay.

The Caspase-3 colorimetric protease assay quantifies caspases based on amino acid sequence. This is a simple and efficient means to analyze caspase activity. This kit is based on the detection of the chromophore p-nitroanilide (pNA), after cleavage from the labeled substrate DEVD-pNA (DEVD is an amino acid sequence Asp-Glu-Val-Asp). Spectrophotometer or a microtiter plate reader at 400 nm or 405 nm can be used to quantify the pNA. Determination of effective increased activity of caspase-3 can be achieved by comparing the amount of pNA absorbed from an apoptotic sample.[30,31]

### Nuclease assay

Nuclease assay technique detects every single RNA molecule from a heterogeneous RNA sample. RNA molecules of any known sequence can be detected even at very low concentration by using this technique.

Degradation of chromatin represents a clear commitment to death;numerous assays have been developed to assess the relevant nucleases involved.

These assays fall into two major categories:

1. Those independent of chromatin structure
2. Those dependent on chromatin structure.

The chromatin-independent assays (plasmid degradation assay and radioactive gel assay) examine the ability to degrade naked DNA are advantageous because of their simplicity and speed and ability to analyze single nucleases or mixtures of nucleases. However, these assays do not mimic the conditions present in normal cells and do not assess the ability of an enzyme to function in apoptosis. In contrast, chromatin structure-dependent assays (nuclear autodigestion and HeLa nuclei assay) present intact chromatin to either endogenous or exogenous enzymes and assess the ability to degrade chromatin in a manner that recapitulates the genomic destruction seen *in vivo* and helps study apoptosis.[32]

## Conclusion

Various apoptosis detection modalities are currently available. Appropriate detection, quantification, determining the stage and validating the observations are the prime requisite of detecting and analyzing apoptosis. Determining the apoptotic index is a good prognostic marker for cancer. Deep understanding of the molecular interlinks between tumorigenesis, apoptosis, and drug targets will provide a strong foundation for new age individualized cancer therapy.

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