In vitro assays for cell death determination

Vladimir Jurišić[1], Vladimir Bumbaširević[2]

SUMMARY

In this paper, we focused on commonly used in vitro assays for estimation of cell death: morphological analyses of cell death, cytotoxic assays based on enzymes activity determination, flow cytometry, and western blot techniques. We discussed advantages and disadvantages of several assays used in the modern research for estimation of cell death.

Key words: *In Vitro; Cell Death; Apoptosis; Necrosis; Flow Cytometry; Blotting, Western; Comet Assay; Lactate Dehydrogenases; Colorimetry*

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[1]School of Medicine, University of Kragujevac, Kragujevac, Serbia,
[2]Institute of Histology, School of Medicine, Belgrade, Serbia

Correspondence to:
Prof. Vladimir Jurišić, School of Medicine, University of Kragujevac, P. Box 124, 34000 Kragujevac, Serbia
vdvd@mailcity.com

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INTRODUCTION

Apoptosis and necrosis are two forms of cell death that have been defined based on distinguishable, morphological criteria. However, these different types of cell death may involve several common signaling and execution mechanisms. Since various stimuli induce both apoptotic and necrotic death, the mode of cell demise seems to be dependent on intracellular factors (1-4). Moreover, recent evidence suggests that identical receptors, signal transduction pathways, and mechanisms of cytotoxicity can be involved in apoptotic or necrotic cell death. It seems that apoptosis and necrosis represent two extremes of a broad spectrum of cell death modes. Cytotoxic pathways and morphological characteristics may be determined by different intracellular factors and external conditions (10-14).

Apoptotic cell death has been implicated in embryonic development, immune system regulation, morphogenesis, in the preservation of tissue homeostasis, and in various disease states (1-3). In multicellular organisms, the mode of cell death has considerable significance extending beyond the fate of the individual cell. Apoptotic cells are efficiently and rapidly phagocytosed, before they lyse and cause inflammation. This facilitates tissue reorganization or reconstitution during tissue development and after cell damage. Moreover, characteristic of apoptotic process is to prevent spread or release of DNA from transformed and virus-infected cells. The apoptosis pathway can be triggered by extra cellular or intracellular signals. Extra-cellular signals include receptor ligands such as TNF and FAS (CD95) molecule (5-9).

Tumor cells use diverse mechanism to escape apoptosis machinery and suppression of apoptosis is known characteristic of cancer growth (8).

Apoptosis can be divided into three phases: initiation, effector, and degradation phase. The initiation phase is largely dependent on a cell type and apoptotic stimulus (e.g., oxidative stress, DNA damage, ion fluctuations, and cytokines). In certain instances, initiation phase may influence the efficacy of the effector and/or degradation phases. In the effector phase, there are activation of proteases, nucleases, and other diffusible intermediaries that participate in the degradation phase of DNA (15,16). Together, the effector and degradation phases promote the ultrastructural features that are suggestive of apoptosis. The genome is usually cleaved into fragments of about 300 and 50 kbp (so-called high molecular weight fragments). Often this is followed by oligonucleosomal DNA fragmentation (*i.e.*, formation of fragments of about n x 180 bp size). Although DNA fragmentation is not the cause of cell death, it seems to be an important feature of apoptosis in relation to the surrounding tissue, and can be used as a diagnostic criterion. Consequently, in the majority of apoptotic models, the interruption of these phases does not confer cell survival, rather it merely deregulates what was to be regulated cell death. Non-caspase proteases like cathepsins, calpains, and granzymes have been implicated as effectors of apoptosis.

Apoptosis estimation *in vitro*

The accumulated knowledge about regulation of apoptosis resulted in assays that may help to understand better the apoptotic process. In this paper, we comment different *in vitro* methods that might help cancer researchers in explanation of dynamic changes during apoptotic and necrotic processes.

CELL VIABILITY ASSAY

Calculation of cell viability and the total number of viable cells (Figure 1) are widely used methods in cell suspension preparation, for cell treatment with toxins, drugs, cytokines and for estimation effects of apoptosis triggering molecules. This is also important step when dose-response effect is evaluated per cell number. In addition, determination of viable cell number is a start point in cell separation protocols regardless the separation method. The most common assays for estimation of cell viability are based on cell membrane integrity and among them dye exclusion assay with trypan blue is widely used in routine laboratory work. Blue stained cells are dead cells and the percentage of viable cells is calculated as ratio of viable (unstained) and total number of enumerated cells (dead and viable cells) (Table 1). Cell counting is commonly done using hemocytometer and classic light microscope (Figure 1).

Table 1. Application of different methods to study cell events during apoptosis

Detection methods	DNA fragmentation	Mitochondria function	Caspase activity	Membrane damage
Light microscopy	+	–	–	+
Fluorescence microscopy	+	+	+	+
Flow cytometry	+	+	+	+
Fluorescence reader	-	+	+	+
Western blot	-	+	+	-

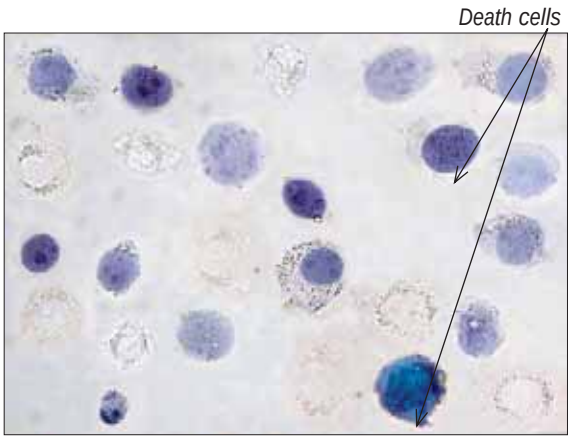


Figure 1. Estimation of cell viability by Tripan blue assay

ELECTRON MICROSCOPY INVESTIGATION OF APOPTOSIS

Apart from classical cytology, electron microscopy (EM) is used for investigation of apoptosis and necrosis in cultured cells. Electron microscopy gives excellent intracellular and ultrastructural cell characteristics on which one can study every stage of apoptosis (Figure 2a, b).

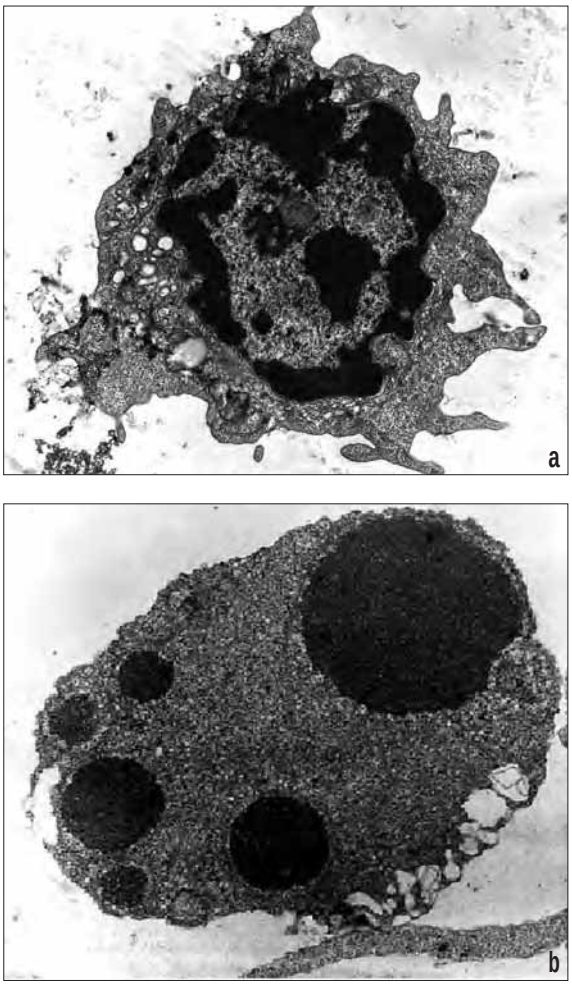


Figure 2. The morphology of the peripheral blood lymphocytes in cell cultures in early (a) and terminal (b) stage of apoptosis (transmission electron microscopy x 100)

Electron microscopy studies simultaneously with classic cytology/histology provide the background for the formulation of the apoptotic cell death concept, which was proposed by Wyllie (13). This concept can be used until today. An apoptotic cell typically undergoes shrinkage (i.e., apoptotic volume is decreased), chromatin condensation, karyorrhexis, and the eventual budding of the plasma membrane into apoptotic bodies. These morphological changes are considered the gold standard for distinguishing this type of cell death (13,17,18). Conversely, oncosis is a passive catastrophic cellular event where marked swelling, aggregate organelle disruption, and plasma membrane blebbing prevail. There is little or no evidence of chromatin remodeling during oncosis, and the cell rapidly succumbs to cytolysis. This cytolysis is end-stage cellular decay that is the defining feature of necrosis. Apoptotic cells will eventually lose plasma membrane integrity and become necrotic *in vitro*. However, this is not believed to occur with high frequency in vivo because apoptotic cells display signals (e.g., the externalization of phosphatidylserine (PS) on their plasma membrane) that encourage their expeditious removal by phagocytosis.

However, EM studies are not recommended for routine work due to expensive equipment and highly trained and experienced personal. Besides, high cell number is required for data interpretation especially when we exactly need to express degree of apoptosis, for example as apoptotic index (number of apoptotic cells per 1000 enumerated cells (19).

ASSAY FOR ESTIMATION OF DNA FRAGMENTATION

One of the hallmarks of apoptosis is DNA fragmentation (13). Commercial apoptosis detection kits (TUNEL assay) enable to study apoptotic cells *in situ* by specific end labeling of DNA fragments. Nucleotides labeled with either digoxigenin or fluorescein are enzymatically added to 3' hydroxyl DNA ends by deoxynucleotidyl transferase (TdT). This enzyme is more sensitive for apoptotic DNA fragmentation than to necrosis, and it is more specific that DNA polymerase. The antidigoxigenin antibody fragment carries either a conjugated reporter enzyme (peroxidase) or fluorescent molecules to the reaction site. The localized peroxidase enzyme then catalytically generates and intense signals from chromogenic substrate that can be observed using light microscopy, while fluorescein can be observed by florescence microscopy or by flow cytometry (20,21).

Large number of DNA fragments that appeared in apoptotic cells result in multitude of 3' hydroxyl termini. This is used to identify apoptotic cells by labeling the 3' hydroxyl ends with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). A substantial number of these sites are available in apoptotic cells providing the basis for the method utilized in the APO-BRDU kits. Data indicated that Br-dUTP is more readily incorporated into the genome of apoptotic cells than fluorescein, biotin or digoxigenin. Non-apoptotic cells do not incorporate significant amount of Br-dUTP.

Comet Assay IV TM developed from Perceptive instrument, Laboratory Company, is widely recommended as an interactive live video-based system for scoring cells subjected to the single cell gel electrophoresis technique (comet assay).

Comet Assay IV incorporates all major measurement parameters that are necessary for estimation of degree of apoptosis (21,22). Once the target number of cells or a particular slide have been scored, data can be saved to Microsoft Excel for the next analyses (Figure 3). Using a high-definition video camera attached to the microscope in the system, Comet Assay IV can transfer a live video picture to computer monitor.

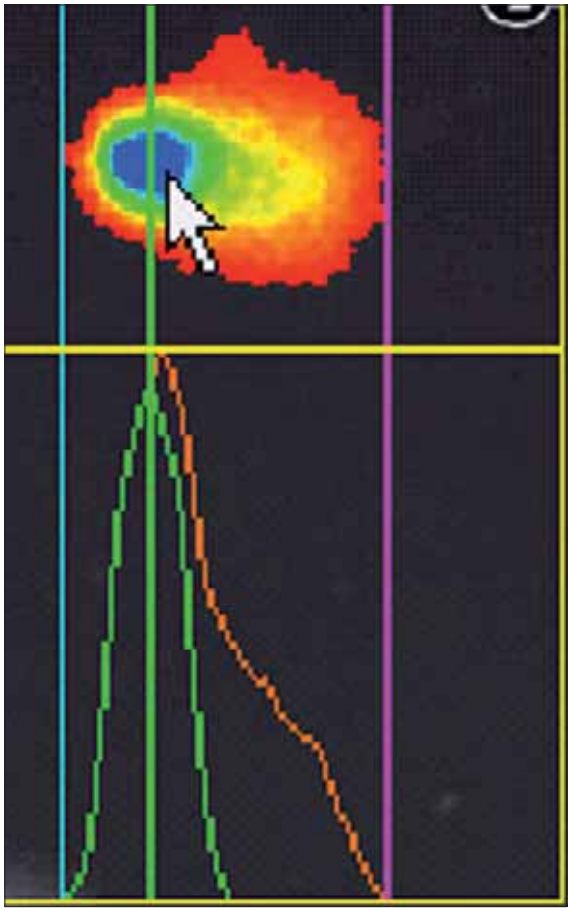


Figure 3. Comet assay

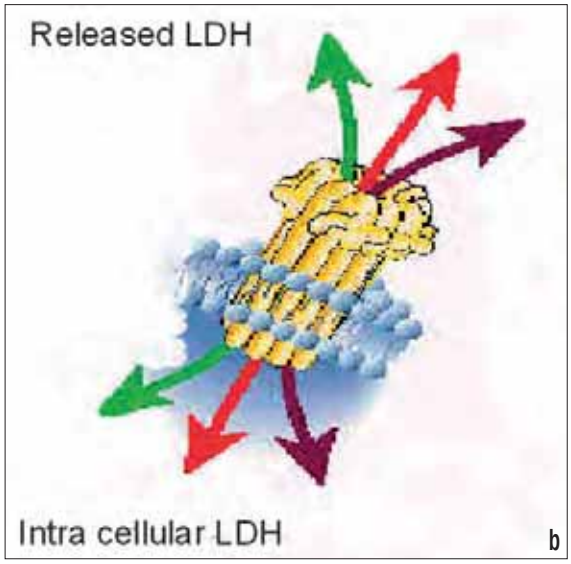


Figure 4. a) Change in absorbance during estimation of LDH release activity by colorimetric assay, b) determination of LDH release activity in extra cellular space (supernatant of cell culture) and into intracellular space (after cell sonification by ultrasound)

COLORIMETRIC ASSAYS FOR STUDY CELL DEATH

The cytotoxic assays described below are the most useful option for cell death investigation for everyday laboratory work, since they are inexpensive, easy for manipulation and obtained data are reproducible and comparable (Figure 4a). Among these colorimetric assays, authors prefer assay based on determination of released intracellular molecule, lactate dehydrogenase (LDH).

LDH is released through the altered cell membrane following cell death process (Figure 4b). The assay principle is based on consideration that tumor cells possess high concentration of intracellular LDH (22-25). In the presence of the drugs or cytokines that trigger cell death receptors superfamily tumor cells undergo apoptosis or necrosis. After cell membrane damage, LDH can be released and thus we detect death cells (25). For exact calculation of percentage of dead cells, it is needed to calculate the intracellular LDH amount in respect to the released LDH amount.

LDH release assay is rapid and very sensitive (26-28). Significant LDH release from cultured cells depends on cell type (tumor or normal), cell number (29) or cell separation process. Cultures of peripheral blood lymphocytes, separated from healthy volunteers or many tumor cells (K562, Raji, HeLa, PC-MDS) after *in vitro* treatments with TNF, showed significant dose-dependent increase in LDH activity (27,30-32). In addition, LDH is mostly released in comparison to other intracellular enzymes, and it is useful since it represents anaerobic type of tumor cell metabolism.

The determination of spontaneous LDH release in cell supernatants is a very appropriate for the estimation of natural killer (NK) cell death in evaluation of innate immunity, safety of vaccine application *in vitro* and *in vivo*, and for virus toxicity on cultured cells (32-35). Using LDH release assay we can detect minimal membrane damage. This assay is also widely recommended as non-radioactive, rapid (2 h versus 4 h) and safety one in replacement of classical radioactive chromium release assay for estimation of NK cell activity,

Sulforodamine B assay (SRB) is other colorimetric assay commonly used for estimation of cell sensitivity to cytotoxic agents. It is based on determination of total protein content in cultured cells before and after drug application. SRB assay can serve to determine the percentage of cell growth inhibition in cultured cells as well as cell percentage of cytotoxicity (36-38).

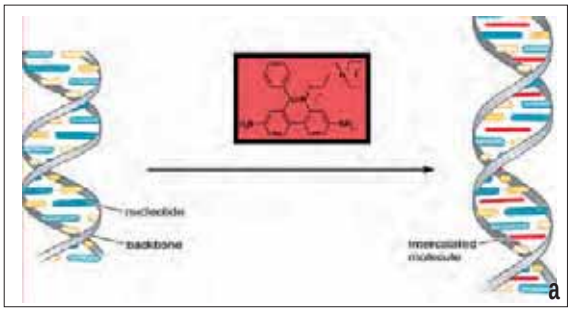
CASPASE ACTIVITY ASSAYS

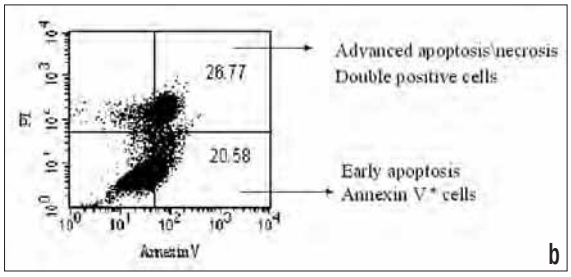
All typical signs of apoptosis are the result of activity of a complex biochemical cascade of events that execute cell proteolysis. Apoptotic signaling mainly converges in the activation of intracellular caspases, a family of cysteine-dependent aspartate-directed proteases, which propagate death signaling by cleaving key cellular proteins. Currently, 14 members of the caspase family have been identified, and 7 of them mediate apoptosis. Several assays were developed to study these molecules.

One assay for caspase detection is based on spectrophotemetric measured such as previously described, but determined chromophore p nitroanilide (pNA) after cleavage from the labeled substrate YVAD-pNA. The pNA light emission can be quantified using a spectrophotometer or micro plate reader. Comparison of the change in absorbance from apoptotic samples with controls allows determination of the fold increase in caspase activity as easy option (39). This assay is semi quantitative but for quantification of caspase activity, we have also flow cytometry or western blot techniques.

ANNEXIN V/PROPIDIUM IODIDE (ANNV/PI) ASSAY

This assay is based on the estimation of cell membrane changes during apoptosis and ability of the protein annexin V to bind to phosphatidylserine exposed on the outer membrane leaflet in apoptotic cells (40, 41). In viable cells, phospahtidilserine is located in the inner membrane leaflet, but upon induction of apoptosis, it is translocated to the outer membrane leaflet and becomes available for annexin V binding. However, phosphatidylserine is also appears on the necrotic cell surface. Using of simultaneous combination of annexin V and propidium iodine (PI) there are different option to discriminate apoptotic from necrotic cells (Figure 5a, b, c). The addition of PI enables that viable (AnnVneg/PIneg), early apoptotic (AnnVpoz/PIneg), late apoptotic (AnnVpoz/ PIpoz) and necrotic (AnnVneg/ PI poz) cells can be distinguished.





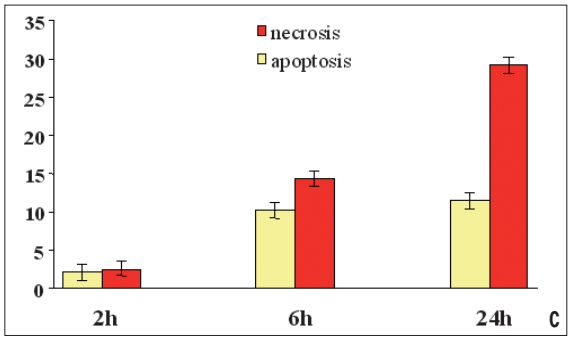


Figure 5. a) Incorporation of PI into DNA of apoptotic cells, b) Increased rate of apoptosis and necrosis (%) determined by flow cytometry using Annexin-V and propidium iodide, c) Increase of rate apoptosis and necrosis in tumor cells induced by TNF in time depending manner calculated by data obtained from flow cytometry

This assay requests flow cytometry for results interpretation. Flow cytometry technique enable expression analysis of several cell surface molecules and have a great application in hematology (40). However using PI/annexin as s double stained system we can also determined viable cells. Data acquisition and analysis by flow cytometry use computer system for determination events on membrane or changes in nucleus on separated flow cells in suspension. The acquisition of events is performed using software. Analysis can be completed after a previously fixed total number of events acquired. Debris cells need to clearly discriminate from nonviable cells.

ANALYSES OF COMPLEX MITOCHONDRIA FUNCTION DURING APOPTOSIS

In healthy cells, the primary function of mitochondria is ATP production (39). This energetic intermediary metabolism is required for maintenance of cellular homeostasis and normal cellular functions. However after a cellular disturbance, the role of mitochondria can change drastically to being a promoter of both necrotic and apoptotic cell death. In an attempt to explain numerous phenomena in mitochondria, again several assays must be performed. Membrane proteins, regulatory proteins (such pro-apoptotic bax and anti-apoptotic bcl-2), chaperone proteins, and other molecules involved in this action can be determined by western blot techniques. Polarization of mitochondria can be detected by fluorescence plate micro assay (42,43). However, electric potential of membrane request electrophysiological investigation by estimation calcium voltage channel function.

TECHNIQUES FOR DETERMINATION OF PROTEINS INVOLVED IN REGULATION OF APOPTOSIS

Western blot is technique that assayed total amount of proteins from investigated cells using electrophoresis methods, and exact confirmation of presence or absence of proteins using immunoassay. In western blot technique, several steps are recognized. Preparation of cytosole proteins, nuclei or nuclear extracts is starting step that request hypotonic cell lyses buffer mixed with protease inhibitors or cell lyses by physical force. The protein concentration of the samples must be determined according to the standard curve (bovine serum albumin as standard) generated in the same setting. Control and experimental samples were run in the same setting to eliminate any assay-to-assay variations. Immunoblotting is the next step and require subjected isolated proteins to SDS-polyacrylamide gel electrophoresis on 16 % poly-acrylamide gels. After electrophoresis, proteins are transferred to membranes in immunoblotting process. The gels are blotted usually with nitrocellulose membranes and stain with Ponceau S red to verify equal loading and transferring of proteins to the membrane in each lane. The membranes must be blocked by non-fat milk in phosphate buffered saline and incubated with respective primary antibody for protein detection. Secondary antibody request further incubation and band are detected by enhanced chemiluminiscence system. Secondary antibodies used in this technique must be conjugated to horseradish peroxidase for chemiluminiscence assay and signals must be developed by chemiluminescent substrate. The signals request visualization by exposing the membranes to X-ray films and digital records of the films need to be captured by a digital laboratory camera (44,45).

Using this assay and appropriate monoclonal antibodies we can determine pro-apoptotic Bcl-2 family members (bid, bad, bax, bag, bak, bcl-xs), anti-apoptotic bcl-2 members (bcl-xL, Bod, Bim, Mcl-1), apoptosis activators (Cytochome c, Caspase 9 and apoptosis inhibitory factor) as well as apoptosis inhibitors (Akt, Bap31, Survivin).

CONCLUSION AND RECOMMENDATION

Different *in vitro* methods for estimation of cell death processes in cell cultures were described (Figure 1). The recommendations from several studies when analyzing cell death process by single method indicate that more assays are necessary in order to better interpretation of data and understanding of complex events on cell membrane, intracellular space or nuclear events during apoptosis and necrosis.

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Conflict of interest

We declare no conflicts of interest.

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