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METHODS FOR THE DETECTION OF APOPTOSIS

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ABSTRACT: Apoptosis is a considered as a critical process regulating several biological processes like differentiation, proliferation, development and maintenance of organs and tissues, proper functioning of immune system and the removal of defective harmful cells. It is also called as "programmed cell death" which is essential for normal metabolism. The process is characterized by different morphological features and changes in biochemical mechanisms. These differences have been exploited for designing the methods for the detection of apoptosis. In our present report, we will give an overview of the apoptosis and various procedures for detecting apoptotic cells. The features, pros and cons of different methods are also discussed.

KEYWORDS: Caspases, Phosphatidyl-serine, TUNEL assay, Annexin, DNA polymerase, Membrane-Blebbing, Hoechst stain, Propidium iodide.

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1.INTRODUCTION

The term "apoptosis" was first of all coined by John Kerr in 1972 (Kerr et al, 1972). It is an extremely regulated phenomenon for maintaining wound repair, developmental, defence mechanisms and normal cellular homeostasis (Susan E, 2007). Morphologically, the characteristic features of apoptotic cell include changes in refractive index of cell followed by cytoplasmic shrinkage, chromatin condensation and loss of membrane phospholipid symmetry (Hengartner, 1997). The normal cells have a phospholipid namely phosphatidyl serine on inner leaflet in their membranes whereas in apoptotic cells, this particular phospholipid flips to the outer leaflet (Williamson, 2011). The protusions in the form of blebs or spikes appear on the cell membrane, which gives apoptotic cell a distinctive appearance. The molecular mechanisms of apoptosis involve a number of steps which

ultimately result in protein cleavage, DNA degradation, membrane asymmetry and formation of apoptotic bodies from the breakdown of cells. Based on these characteristic features of apoptotic cells, different methods have been developed to understand and detect apoptosis. Due to its role in the progression of several diseases, it is considered among the hot topics of biological research (Sue et al, 2006). Normally, it is required for the removal of unwanted cells in the multi-cellular organisms, but the defects in the regulation of apoptosis can result in adverse consequences. The infecting agents and often tumour cells evade the normal induction of apoptosis as a strategy of increased survival in host. However, in some neurological disorders there are excessive levels of apoptosis, thereby, explaining the need to understand this critical phenomenon.

1.1. Apoptotic Mediators

In 1986, Robert Horvitz while studying the developmental processes of C. elegans (nematode worms) discovered mutation in ced-3 gene due to which there was no loss of cells to apoptosis. This gene coded for proteins which played an important role in the process of apoptosis in C.elegans (Ellis & Horvitz, 1986). This further led to the recognition and discovery of homologous protein family, Caspases in mammals. Caspases are a class of highly conserved protein family involved primarily in apoptosis and inflammation. They are expressed as inactive zymogens consisting of one large subunit, one small subunit and an N-terminal prodomain. Upon removal of prodomain by cleaving the zymogen at specific aspartic acid residue, the inactive zymogen gains full activity. The active caspase protein comprises of a tetramer of two heterodimers. They are the proteases with Cysteine residue at the catalytic site and cleave number of proteins for triggering the process of cell death. They cleave Focal Adhesion Kinase (FAK), cytoskeletal proteins, lamins etc which leads to the detachment of apoptotic cell from its neighbours, changes in cell shape and disassembly of the nuclear envelope and shrinkage of nucleus. A specific DNase known as Caspase Activated DNase (CAD) elicits DNA fragmentation through activation by Caspases (Cohen, 1997). The Caspases are categorised as initiators (Caspase 2, 8, 9, 10), executioners (Caspase 3,6,7) and inflammatory Caspases (Caspase 1,4,5) (Hengartner ,1992). In humans, Caspase 8 and Caspase 9 initiate the apoptosis and Caspase 3 executes and commits the cell towards apoptotic fate. Another important mediator of apoptosis includes the Bcl-2 protein family. On the basis of structure, the Bcl-2 protein family are divided into three groups; the multiregional pro-apoptotic proteins that permeabilize the mitochondrial outer membrane, the anti-apoptotic proteins that hinder the process and the BH3 proteins that activate the pore-forming class members (Daniel, 2003). Bid and Bax are the pro-apoptotic members whereas Bcl-w, Bcl-2 and Bcl-XL are the counter apoptotic members of Bcl-2 protein family. The fate of a cell is determined by the critical and delicate balance between pro-apoptotic and anti-apoptotic proteins inside a cell (Wajant, 2003).

1.2. Apoptotic Activation

The stimulus for triggering of apoptosis can be either internal or external and thereby the signalling cascades activating apoptosis are classified into intrinsic and extrinsic pathway. The extrinsic stimulus constitutes the proteins secreted from the cells of the immune system e.g. TNF- α (Tumor necrosis factor alpha). It is a protein secreted in response to various reasons like viral infections, elevated temperatures, exposure to harmful radiations and toxic chemical agents. The binding of TNF- α to its transmembrane receptor TNFR1 leads to conformational changes in the cytoplasmic domain of TNF-α receptor, thereby recruiting several proteins. The inactive caspase-8 is the last protein that gets recruited and activated due to proteolytic cleavage. The active caspase-8 known as initiator caspase, leads to the activation of downstream Caspases that trigger the self-destruction of cell. The other death receptors include the TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1) and Fas receptor which are activated by the ligands TRAIL and Fas respectively (Hengartner, 2000). The intrinsic stimulus constitutes the oxidative stress, hypoxia, DNA damage and high Ca2+ concentration. The intrinsic pathway is mediated and regulated by Bcl-2 family of proteins. One of the proapoptotic members of Bcl-2 protein family, Bax, translocates from cytosol to mitochondrial membrane. This event leads to increased permeability of the membrane and release of mitochondrial proteins. The other prominent protein secreted is the cytochrome c. Once released cytochrome c becomes part of a protein complex known as apoptosome. The multiprotein compqlex comprises of inactive caspase-9 which gets activated by joining the complex rather than proteolytic cleavage. The active caspase-9 activates the downstream Caspases which lead to apoptosis. The Extrinsic and Intrinsic pathways converge by activating the executional Caspases which destroy the cellular targets. Although the pathways are activated by different stimuli but they are linked and influence each other (Zou, 1997; Igney, 2001).

1.3. Assays for Detecting Apoptosis

The measurement of apoptosis is vital to assess the cytotoxicity of medication, to identify apoptosis and to elucidate the mechanism that prompts apoptosis by different compounds. The indicators of apoptosis such as cytochrome c are used for monitoring apoptosis. Caspases are among the best targets for detecting apoptosis within cells as they become activated during apoptosis. The highlights like DNA fragmentation and loss of membrane symmetry can likewise be used to determine apoptosis. The selection of a method relies on various parameters like the type and number of cells, the pathway which induces the apoptosis and the exact method of analysis. Some of the commonly used utilised methods for the detection of apoptosis are mentioned and briefly discussed.

1.3.1. Analysis of Cytomorphological alterations

Cytomorphological modifications because of apoptosis involve nuclear and cytoplasmic condensation of cells. For detecting these changes, cells are treated with haematoxylin and eosin dyes and visualised under light microscopy. The single apoptotic cells can be detected by this method but

it requires confirmation by other techniques as well. Toluidine blue or methylene blue is also used for revealing intensely stained apoptotic cells by standard light microscopy. The limitations of the method arise due to the false positives for healthy cells having dense intracellular granules.

1.3.2. Monitoring DNA fragmentation

One of the characteristic features of apoptosis is DNA fragmentation and forms the basis of a number of methods for detection of apoptosis. The chromosomes are first cleaved into large fragments followed by cleavage of DNA into 180-200bp fragments by the action of Ca2+ and Mg2+ dependent endonucleases. The cleaved DNA fragments can be seen as a ladder on agarose gel electrophoresis. However, this way of visualisation is suitable only when large number of cells is involved in apoptosis. The alternate methods are used when only a few cells are apoptotic. TUNEL is one of the widely used assays for detecting apoptosis. The in situ labelling of DNA breaks on dufferent types of tissues was first done by Garvieli, Sherman and Bensasson (Gavrieli, 1992). In this assay, the terminal deoxynucleotidyltransferase (TdT) is used to label the fragmented DNA breaks. The TdT polymerase catalyzes the addition of labelled deoxynucleotides to the 3'OH ends of DNA. The nucleotides are usually labelled with fluorescein, biotin or DIG and detected by standard immuno-fluorescent and immuno-histochemical techniques. The biotin labelled nucleotides are detected by using avidin conjugated to a reporter (e.g. alkaline phosphatase), the nucleotides labelled with FITC are directly detected by immuno-fluorescence and the DIG labelled nucleotides are detected by conjugated anti-DIG secondary antibody. The adherent cells, smears, cytopsin preparations, cryopreserved tissue sections are first fixed in paraformaldehyde solutions, washed with phosphate buffer saline and then permeabilised (Triton X-100 and sodium citrate). This is followed by the addition of TUNEL reaction mixture and finally the analysis of samples is done depending upon the type of labelling of nucleotides (Grasl-Kraupp, 1995). TUNEL assay is considered best for detection of apoptosis because it works on a various cell types. However, the assay is critical to fixation and thus makes sample size critical. It is expensive and time consuming. Identification of apoptosis by TUNEL assay is not sufficient because the chromosomal DNA degradation also occurs in necrosis and therefore the assay is not specific.Staining of cells y dyes like Hoechst 33342 and DAP1 have also been used for the detection of apoptosis. These dyes become highly fluorescent upon binding to DNA and thus make the chromatin condensation of apoptotic cells readily visible. The dyes like Propidium Iodide have also been used in conjugation with other methods for the determination of apoptosis. The dye is impermeable to membrane and cannot pass the viable cells, thereby stains dead cells only. Propidium Iodide intercalates between the bases and its fluorescence gets enhanced on binding to DNA. The dye is suitable for flow cytometry, fluorescence microscopy, fluorimetry etc (Zink, 2003).

1.3.3. Mitochondrial markers of Apoptosis

The disruption of active mitochondria is a characteristic feature of early stages of apoptosis. It includes alterations in the membrane potential and oxidation-reduction potential of mitochondria.

These changes are presumed to occur due to change in mitochondrial membrane permeability allowing passage of molecules across it. The common method for detecting the change in mitochondrial membrane permeability involves a fluorescent lipophillic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). The dye has been used as an indicator of mitochondrial membrane permeability in a variety of cell types as well as in tissues and isolated mitochondria (Cossariza, 1993). In monomeric form, JC-1 emits at 527nm whereas the aggregated form is associated with a significant shift in emission (590nm).The dye accumulates inside mitochondria in non apoptotic cells, remain as aggregates, and thereby emit bright red fluorescence. However, in apoptotic cells, the mitochondrial membrane does not maintain electrochemical gradient and leads to diffusion of dye into the cytoplasm and therefore green fluorescence specific for monomeric forms is emitted. JC-1 staining can be used for both suspension as well as adherent cells. The cells are first seeded depending upon the recommended density for a particular cell type. For suspension cells, the staining solution (JC-1 stain dissolved in DMSO) and growth media are mixed and then the cells are incubated at 37°C in a humidified atmosphere containing 5% CO2. The cells are then centrifuged and pellet is resuspended in staining buffer. The stained cells are then observed by fluorescence microscopy or by flow cytometric assay and measured by fluorimetric assay. For adherent cells, firstly the media is aspirated out and then the cells are incubated with staining solution. Then, the staining solution is aspirated out, cells are washed with buffer or growth media and visualised under fluorescence microscope. Since, the membrane potential is sensitive to changes in PH and temperature; therefore the reagents should be carefully checked. The dye is sensitive to light and thus incubations need to be done in dark and the samples should be analysed promptly after completion of staining. Apart from JC-1, other dyes such as rhodamine-123 and DiOC6 have also been used to estimate the apoptotic changes but they are not reliable due to less sensitivity and specificity. One of the critical players of apoptosis which has been largely used for the detection of apoptosis is the Cytochrome c. It is a water soluble protein located in the intermembrane space of mitochondria. The stimulation of apoptosis leads to the release of cytochrome c from mitochondria into the cytosol, where it forms a complex with Apaf-1 and activates Caspase 9. This leads to the activation of Caspase 3 and the downstream executioner Caspases. The translocation of cytochrome c from mitochondria towards cytosol paves way for the detection of apoptosis. The mitochondrial and cytosolic fractions are isolated and western blotting technique using cytochrome c antibody is used to study its translocation and thereby the event of apoptosis (Li et al, 1997). Immunohistochemistry and ELISA can also be done for the detection of cytochrome c. BCl-2 protein family which have a critical role in apoptosis are also detected by western blotting and immunohistochemistry.To monitor the integrity of outer mitochondrial membrane, cytochrome c oxidase assay is carried out. Cytochrome c oxidase is an enzyme located on the inner mitochondrial membrane and involved in coupling of electron transport with oxidative phosphorylation. The assay is based on the decline in

absorbance of ferrocytochrome to ferricytochrome due to its oxidation caused by cytochrome c oxidase. The assay is carried out in presence and absence of detergent (n-dodecyl β-D-maltoside). The detergent at low concentrations maintains the cytochrome c oxidase dimer in solution. The ratio between the cytochrome c oxidase activity in presence and absence of detergent is a measure of integrity of outer mitochondrial membrane, since the membrane is the barrier for the entry of cytochrome c into organelle. The assay requires the use of freshly prepared samples as the frozen tissues may cause rupture of sub cellular organelles.

1.3.4. Measuring Changes in Cell membrane to detect apoptosis

The plasma membrane of cells have asymmetric distribution of phospholipids was first of all reported in erythrocytes. During apoptosis, the asymmetry of phospholipids on cell membrane is lost due to the flipping of Phosphatidylserine towards outer leaflet of membrane (Zachowski, 1993). Detecting the change in phospholipid asymmetry is one of the ways to detect apoptosis. Annexin-V is a phospholipid binding protein that has a high affinity for Phosphatidylserine and conjugating Annexin V to a fluorescent molecule or dye can be used to label apoptotic cells. Koopman *et al* first described a method of using Annexin V labelled to applied hapten (i.e. FITC/ Biotin) to detect apoptosis. The labelled Annexin V binds to the Phosphatidylserine residues in presence of Ca^{2+}. Annexin V cannot penetrate the phospholipid bilayer and as such does not bind intact live cells. In dead cells, the integrity of plasma membrane is lost and the labelled Annexin V binds to inner leaflet of membrane. However, to discriminate between apoptotic and dead cells, DNA stain such as propidium iodide can be added. Thus the apoptotic, viable and dead cells can be distinguished on the basis of the double labelling for Propidium Iodide and Annexin V and subsequently analysed by flow cytometry and fluorescence microscopy. However, for quantification of apoptotic cells, flow cytometry can be used by using a cell suspension prepared from cells or tissues. The assay has additionally been utilised for identifying the apoptotic cells in situ. The biotin labelled Annexin is infused into mice and then followed by dissection and formalin fixing of tissues. The tissue sections are treated with streptavidin conjugated peroxidase and finally visualised. Consequently, the apoptosis could be detected even in developing mouse embryos by this technique. The phosphatidyl serine exposure is a universal phenomenon that occurs amid early apoptosis preceding the detection of DNA strand fragmentation, providing advantage for detection of apoptosis by Annexin V assay. The assay is quick, simple, sensitive and independent of species or apoptosis inducing systems. However, the assay cannot be used for the cells which express large level of Phosphatidylserine on their outer membrane (Manon et al, 1998).

1.3.5. Detection of apoptosis by monitoring Caspase Activity:

The activation of Caspases is profoundly regulated by transcription and by anti-apoptotic polypeptides and thus, the detection of Caspase activity is among the best strategies for detecting apoptosis (Earnest et al, 1999). Analysing procaspase processing by Immunoblotting, analysing

enzyme activity by cleaving synthetic substrates and the Immunoblotting examination of cleavage of Caspase substrates are among the different methods for the assurance of Caspase activation. One of the important methods for analysing the activation of Caspases involves the detection of their target molecule Poly ADP-ribose polymerase (PARP) by Immunoblotting (Lazebnik, 1997). PARP is a nuclear enzyme involved in DNA repair and is cleaved by Caspase 3 during apoptosis. The Caspase activity assays are among the commonly used methods for the identification of apoptosis and include the use of synthetic substrates. The technique was first described by Pennington and Thornberry. The tetrapeptide sequence mimicking the specific cleavage sites of Caspases are synthesised and conjugated to a detectable entity (chromophore or fluorophore).The Caspase activity assays are colorimetric, luminometric or fluorimetric relting on the sort of tetrapeptide substrate utilised. For luminometric Caspase activity assays, the substrates used are Z-LETD-aminoluceferin (Caspase 8 substrate), Z-DEVD-aminolucefrin (Caspase 3/7 substrate), Z-LETD-aminoluciferin (Caspase 9 substrate) and Z-VEID-aminoluciferin (Caspase 6 substrate). The enzymes used are the luciferases which catalyze the reaction and lumniscence is emitted. The buffers are optimised for monitoring specific Caspase activity. When the Caspases are inactive, the substrates are not cleaved and thus no light is produced. However, upon cleavage of substrate by active Caspase, light is produced which is proportional to the Caspase activity. For colorimetric detection of Caspase activity, the substrates used are labelled with chromophore p-nitroaniline. The chromogen is released out upon cleavage by Caspase and produces yellow colour which is then measured spectrophotometrically at 405nm.The colorimetric detection of Caspase 3 activity provides quantitative measurement of protease activity which is a regulatory event in the programmed cell death process. The substrates like profluorescent DEVD peptide-rhodamine 110 are used for the fluorescent detection of Caspase 3/7 activity. The substrates mixed with buffers is added directly to the culture dish and incubated. The cells are permeabilised so that they release the Caspase and the fluorescent product proportional to the Caspase activity in the sample gets accumulated (Green, 2000). The enzyme activity assays are sensitive to numbers of components and therefore, optimal PH, salt concentration and optimal buffer composition should be maintained for efficiently carrying out the assay.

CONCLUDING REMARKS

The detection of apoptosis in *in vitro* studies is an important cytochemical technique. The methods developed are based on the properties associated with the apoptotic cell. The best way is to use more than one method for the detection of apoptosis. One of them should detect an early apoptotic event whereas other an executional event. However, multiplexing more than one assay can provide better insight of the process and eliminate the need to repeat the work.

CONFLICT OF INTEREST

None Declare

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