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Apoptotic Programmed Cell Death in Cancer and The Modern Methods for Detection of Apoptotic Cells: A Mini Review.

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ABSTRACT

Apoptosis is a process of a programmed cell death in all mammalian cells. Gaining understanding into the modern methods for detection of apoptotic cells will let the development of therapeutic agents with the properties to induced apoptotic cells death in cancer therapy. In this context, modern methods for detection of apoptotic cells play an important role to characterize the dying cells. In the present review, we outline the modern methods that are based on morphological analysis, flow cytometry analysis, cell quantification and mitochondrial membrane potential (Δψm) for detection of apoptotic cells death. We also present the basic principle of apoptotic cells death and related latest information in this review.

Keywords: apoptotic, cell death, cancer.

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INTRODUCTION

Apoptosis was a term adopted from the Greek, denoting “dropping off”, an adumbration likely to the falling of leaves from trees in autumn. The word received its great endurance when Kerr et al. [1] recount the plight upon which a cell is initiated by a number of stimuli in directing itself in pursue of death.

Apoptosis is an indicative of a number of morphological changes that witness cell shrinkage, membrane blabbing, nuclear fragmentation and chromatin condensation [2] [3]. Even though this apoptosis is extremely coordinated, still, its role in both physiology and pathology, is subtly controvertible [4] [5], for an instance, the genetic regulators for apoptosis if disrupted by mutation becomes a great contributor to a number of human diseases stretching from neurodegenerative disorders to cancers [6].

Morphological Changes in Apoptosis

In despite of cell type and species, the cellular structure alterations pertaining to nucleus and cytoplasm regions are astonishingly comparable and similar during apoptosis [7] [8]. Often enough, the cells arrives to its final state of cellular fragmentation, several hours after the commencement of programmed cell death, yet the time taken is contingent upon cell type, stimulus and apoptotic pathway as described in Figure 1 [9].

During apoptosis, the cells contract to appear round in shape, followed by decrement in cellular volume and the withdrawal of psedopods. Subsequent alteration will beget in the nucleus uncovering chromatin condensation and nuclear fragmentation, the very hallmark of apoptotic morphology occurring in nucleus [10] [11].

The chromatin condenses around the membrane circumference of the nucleus, generally resembling a crescent or ring-like structure. Typically, the chromatin thickens to the point where it ruptures within the confinement of the cell membrane, a prevailing feature known as karyorrhexis [12]. The plasma membrane will remain integral at every point of apoptosis event.

Shortly, the last phase of apoptosis divulges its vulnerability to membrane blabbing, cellular ultrastucture alteration and loss in membrane integrity [10] [11]. Given the normal physiology, the apoptotic cells will be engulfed by phagocytic cells (macrophages and neutrophiles) before the occurrence of apoptotic bodies.

The artificial cell culture environment, conversely do not facilitate the remnants of apoptotic cells to be phagocytosed, thereby perceives the disintegration of apoptotic cells into necrosis-like state, a condition defined as secondary necrosis [9].

Biochemical Changes in Apoptosis

There are three conspicuous biochemical modifications transpiring in apoptosis: 1) caspase activation 2) DNA and protein degradation and 3) membrane alteration and phagocytic cells recognition [13]. Naturally, an antecedent of apoptosis is imputed to the eversion of phosphatidylserine (PS) from the inner layer to the outer layer of cell membrane. This expedites the initiation of phagocytosis recognition without the involvement of pro-inflammatory cellular components [14].

Thereafter, DNA is degraded into large 50 to 300 kilobase pieces, followed by actions from end nucleases to further disintegrate these DNA into oligonucleosomes in multiples of 180 to 200 base pairs. Though this a distinctive feature of apoptosis, a simple test of DNA ladder in gel electrophoresis might not represent the best outcome, as necrotic cells shares the same consequence of DNA fragmentation too [15].

The enzyme caspase is another addition to apoptosis features upon where the alphabet “c” from the word caspase indicates cystein protease while the “aspase” refers to the enzymes capability to cleave after aspartic acid residues [13]. Caspases which are triggered to an active state will drift to excise fundamental elements such as proteins, nuclear scaffold, and cytoskeleton and even prompt DNAse to accelerate nuclear DNA degradation [16].

It is to be highlighted that, biochemical analyses of DNA fragmentation and caspase activation should not be solely contemplated as apoptosis, as these signs are capable of eventuating without the favour of caspase or oligonucleosomal DNA fragmentation [11]. The Nomenclature Committee on Cell Death (NCCD) recommended that categorizing of cell death procedures should be based upon morphology criteria alone due to existing vague equivalence between ultra structure changes and biochemical cell death analyses [17].

APOPTOSIS DETECTION METHOD

Detection of Apoptosis through Morphological Analysis

Apoptosis has earned disparate methods to uncover their discrete appearance in cells and one of the most advantageous methods is via electron microscopy observation. The finest revelation of apoptosis within the array of tissues and physiological state has been construed with the help of investigations carried out by Kerr et al. [1] and Wyllie et al. [2].

The parameter of cell death following treatment with definite drugs every so often marks the experimental endpoint and while apoptotic morphology is concerned; transmission and scanning electron microscope seems requisite. The samples are fixed using osmium tetra oxide before being inserted into tiny resins. The resins containing samples are then sliced into ultra thin segments only to be finally stained with uranyl acetate (UA) and lead citrate (LC) prior to transmission electron microscope analysis.

UA delivers the highest electron density with better image and contrast by fastening itself to proteins, ribosomes, membranes, lipids and nucleic acids. The LC however augments the contrasting effects across ribosomes, lipid membranes, cytoskeleton and cytoplasmic compartments. Hence, the LC staining is employed soon after UA [18].

Scanning electron microscopy has also been used to examine apoptotic cells. Their characteristic rounded-up phenotype is well represented by this imaging modality [2] [19].

Apoptosis can be distinguished via Giemsa stain, in reference to cellular morphology. Giemsa, an interfusion of methylene blue, eosin and Azure B, trails after DNA phosphate groups to find itself adhesive to adenine-thymine rich DNA region [20]. The periphery of plasma membrane and the nucleic region are more flawlessly observable through light microscope [21].

The HoloMonitor, a quantitative phase contrast microscope is a method modelled to compute the phase shift of live cells or apoptotic cells in their habitual culture vessel. This is considered as an excellent method since these cells are not perturbed by trypsin or stained for their features to be visible. The microscope devises a phase shift image of cells corresponding to measured phase shift giving away coloured images that respond congruously with the height and thickness of cells [22].

Detection of Apoptosis via Flow Cytometry Analysis

Flow cytometry is an instrument discovery of laser-based and biophysical technology that can detect cells in a stream of fluid when they pass by an electronic detection apparatus. It exposes multi-parametic analysis pertaining to the physical and chemical properties of apoptotic features in cells[23].

The emanation of fluorochromes such as Annexin V-FITC and propidium iodide (PI) facilitates the accuracy of the cell detection. The dye PI binds by interpolating between DNA or RNA bases and once fixing itself to nucleic acids, its fluorescence impels to enhance up 20 to 30 fold [24]. The Annexin V labelled in association of FITC (fluorescent substance) bounds to phosphatidyserine (PS). PS, a membrane positioned under natural circumstance lies within the cytoplasmic milieu of typical cells but gets everted to the cell outer surface upon apoptosis. The displacement of PS extricates the visualization of apoptosis apart from normal cells [15] [25] [26].

Nevertheless, this technique is not only pertinent for apoptotic cells, but also applicatory for the determination of necrotic cells in the interest of Annexin V binding to PS from the inside of damaged cell membranes of necrotic cells [27]. A double staining has been proposed to serve the intention of discriminating

apoptotic from the necrotic ones. Both Annexin V and PI will be introduced to the mixture of heterogeneous cells, as a result, apoptotic cells will be stained by Annexin V, while necrotic cells will promote dual staining of Annexin V and PI [28].

The Usage of Flow Cytometry in Cell Quantification

The flow cytometry implements a biophysical technology based on laser and impedance which is utilized completely to quantify individual cells while classifying them in accordance to their biological markers. The cells are first mixed together in a liquid which is then channeled directly into an apparatus that electronically recognizes them. Here, thousands of cells are sorted in regard to their physical and chemical properties per second permitting concurrent multi-parametric analysis.

A flow cytometry's function is analogue to a microscope. Apart from producing observable images, the flow cytometry generates automated measurable parameters for large number of cells. The cytometry is composed of five major constituent; (1) a flow cell that arranges cells into a single line to stream across a light beam, (2) the light beam contributes as a sensing device comprised of optical lamps and lasers, (3) a detector that concerts the measurement into forward scattered light (FSC) and side-scattered light (SSC), (4) a system amplifier which amplifies the extend of signal mechanical qualities and (5) a computer that serves for interpretation of signal analysis [29].

The data perceiving technique from flow cytometer is referred to as 'acquisition' and is facilitated with the use of a computer and software that ensures the accuracy of the data [30]. The data received is scaled and converted into single or two dimensional dot plots where the distribution of the dots are sorted on the basis of fluorescence intensity and separated into subsets (defined as gates). The plots are represented on logarithmic scales to avoid overlapping spectra emission caused by the presence of two or more dyes and also signals received from the detector is subjected to compensation prior to analysis [30].

The study herein incorporated analysis performed using the fluorescence-activated cell sorting (FACS), an advanced technology deriving from flow cytometry. The technique employed in FACS not only similar to the flow cytometry but is also enhanced in terms of obtaining data in much faster and accurate manner [31].

Detection of Apoptosis via Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Apoptotic stimulability is a key lead to $\Delta\Psi_m$ abatement [32] [33] [34] that may ensue during oxidizable substrate deficiency in mitochondria, respiration obstruction or inner membrane uncoupling [35] [36]. The mitochondrion houses a set of proteins that participate throughout the apoptotic mechanism; caspases and cytochrome c. The caspases fit in as members of cysteine proteases family, the focal point inducement of several apoptosis processes [37] [38], while emancipation of cytochrome c from the intermembrane space into the cytoplasm actuate downstream caspases through apoptosome incitation [14] [39].

The establishment of a recent flow cytometry technique in computing $\Delta\Psi_m$ together with the employment of fluorescent lipophilic substrates connotes apoptosis. The healthy cells uptake these lipophilic molecules to constitute agglomerates that emits bright red fluorescence. On the other hand, apoptotic cells, liberates the lipophilic molecules back into cytoplasmic region due to reduced $\Delta\Psi_m$, giving out green fluorescence, the very outcome of monomeric molecules [28].

Necrosis

In the beginning, the term necrosis was thought to be the last stage of irrevocable tissue impairment, engaging only upon cells that have already died [12]. Efficaciously over the years, the definition of necrosis has ameliorated to a passive form of cell death unlike apoptosis, but rather accidental without the perceptibility of convoluted regulative mechanisms. Factors such as heat stress or toxic agents are capable of impelling necrotic cell death as well as in many ways provoke apoptotic cell death [12] [40]. The deciding dynamics concerning the fate as either apoptosis or necrosis relies upon the degree of injury and the level of energy within a cell. Altered or reduced ATP generations often shove cell death in the direction of necrosis [41] [42].

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Necrosis appears to be a set of distinguished characteristics affecting the morphology of these dying cells as illustrated as in Figure 1. The initial response to necrosis is placed contingent on the cell membrane that emerges permeable; as a result, organelles appear dilated while endoplasmic reticulum loses its ribosome. Chromatin condensation only arises in certain cases and nucleus fragmentation occurs late during the stage. Pyknosis and nuclei disintegration are not typical features eventuating in necrotic cell death. Nonetheless, the outflow of hydrolytic enzymes and other cellular components elicit massive inflammation on adjacent cells and tissue.

There are two different forms of necrotic cell death classified mainly on the morphology and participation of lysosomes; autophagic and non-lysosomal degeneration [43]. The autophagic cell death is pecularized with distinctive nature of vacuoles filled with cellular traces bountifully scattered within the cytoplasm region while the non-lysosomal cell death exhibits unequivocal dilation of organelles, empty spaces formation without the involvement of lysosomes. Apprehending the fact that dying necrotic cells develop higher membrane permeability at the earliest stage, as opposed to apoptosis, researchers had ferret out a technique to detect them. Due to the mitigated membrane permeability, tiny charged molecules that generally excluded by the cell membrane will then be directed into the cell.

Similarly, the cell then uptakes PI, mono- or dimeric cyanine nucleic acid dyes, such as YOPRO-1, in which these dyes possess greater affinity to DNA and RNA, hence a suitable correlation was uncovered between the dye binding and the outcome of their fluorescence intensity [44].

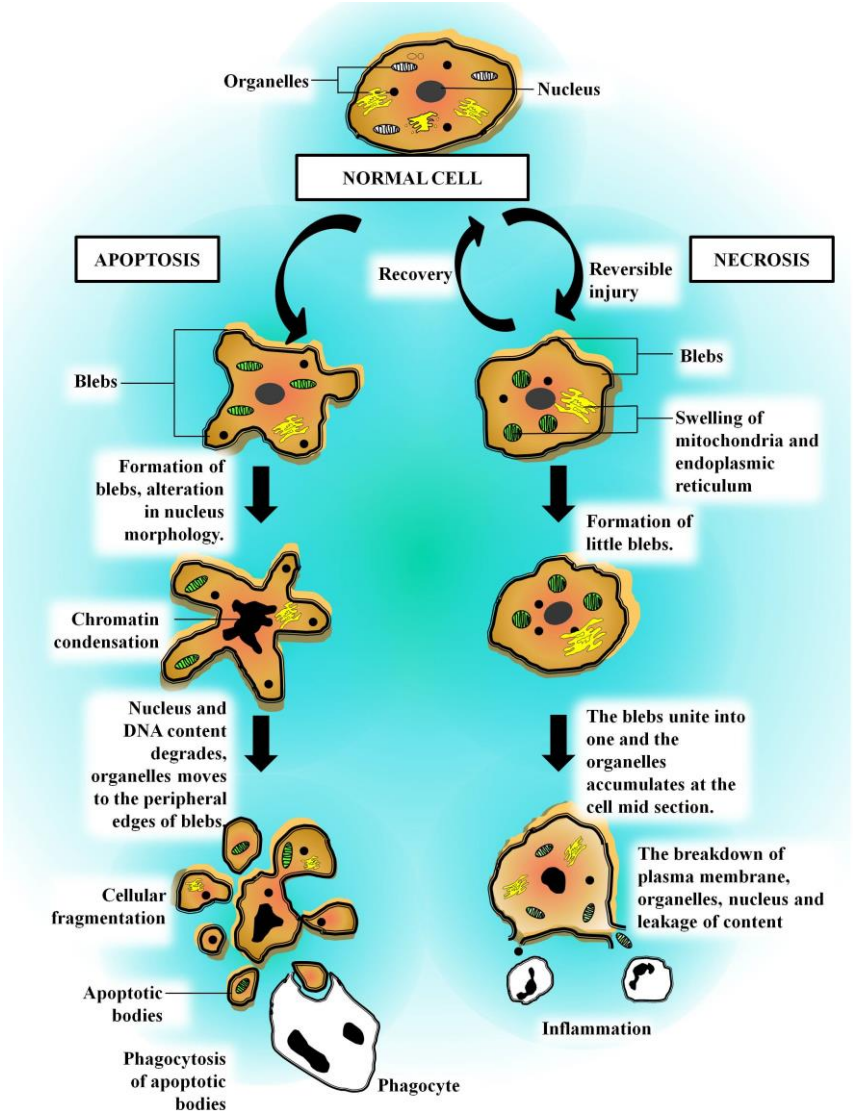

Figure 1: The morphological differences between apoptosis and necrosis mechanisms.

Cell Cycle

The cell cycle machinery incorporates a set of events that is applied conscientiously during cell replication. Eukaryotes submit to homeostatic balance between the process of cell death and cell proliferation to sustain the intricate network of tissues while acclimating to changing environment conditions.

With the intention of achieving a balance, there must be a certain linkage uniting cell cycle and programmed cell death together through a controlling set of components [45].

Cell Cycle and Apoptosis

Cell cycle is a comprehensive study of four phases; G1 phase, S phase, G2 phase and the final M phase. Genetic materials are conveyed from one cell to another cell and this event necessitates genetic materials to be duplicated in the course of S phase, while the M phase governs the explicit formation of two daughter cells. Both phases are unequivocally imperative for the identical regeneration of a cell without genetic defectiveness. The cell cycle machinery is as such that M phase ensues only upon the completion of S phase. There are two prefatory phases existing between S and M phases.

The G1 phase is positioned before the S phase while the G2 phase stands in between S and M phase. Differentiating cells often egress away from G1 phase into a dormant state, represented as G0 phase [46]. The regulation of cell cycle is mediated by two substantial proteins, namely the cyclin (A, B, D, E) and cyclin dependent kinases (cdks). These proteins have to comply with each other through phosporylation to establish cyclin cdk complexes. When the concentration of these complexes becomes commensurable, the checkpoints endorse the cell to move forward into the subsequent phase.

The cell cycle will come to a halt if the concentration as well as the complexes activity were to perturb, for example, by silencing these complexes, the cell cycle ceases and imparts apoptosis. Additionally, there are also other proteins; p53 and Rb proteins that possess underlying roles in the cell cycle regulation. The p53 protein is coded by p53 tumor suppressor gene, which takes control upon the recognition of DNA impairment during c-radiation or chemotherapy [47] [48]. The p53 works by triggering the expression of p21 gene which sequentially arrests the progression of cell cycle by blocking the performance of cyclin cdk complexes. At higher concentration of p53, initiation of BAX transcripts takes place eventuating in apoptosis induction [47]. The proto-oncogene c-myc also impacts the cell division by eliciting p53 dependent apoptosis in murine embryonic fibroblast, a good indication of possible link between p53, c-myc and mitochondrial system [49].

The retinoblastoma (Rb) protein, a tumor suppressor comparably binds to specific numbers of transcription factors to inhibit DNA replication while in another stance, the cyclin cdk complexes simply phosphorylates these proteins freeing Rb from the transcription factors, ensuing the cell to proceed into subsequent phase [50].

Reactive Oxygen Species (ROS)

Cancer cells are reputable for mitochondrial dysfunction, high cellular receptor signalling, elevated metabolic, proxisome, oncogene, oxidases, cyclo-oxygenases, lipoxigenases and thymidine phosphorylase activity [51] [52] [53].

The system of a typical aerobic organism expels reactive oxygen species (ROS) as consequence of oxidative phosphorylation, which can be recognised as hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet}-$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). The electron transport chain found along the inner membrane of mitochondria accommodates five major protein complexes known as complexes (I, II, III, and IV) and ATP syntheses. Nearly 80% of superoxide formed at complexes I and III are liberated into the intermembrane space while another 20% are released into the mitochondrial matrix [54]. The transition pores positioned on mitochondria usually facilitates molecular exchanges and at the same time discharges these superoxides into cell's cytoplasm milieu [55] [56]. This superoxide's eventually become converted into H_2O_2 that act as secondary messengers with a higher permeability towards any cellular membranes [57].

Customarily, the injurious effects caused by superoxide require abrogative action by antioxidant. However, when the level of superoxide exceeds antioxidants, the disproportion caused derives to a state known as oxidative stress. The endurance of oxidative stress induces deterioration at the molecular level which also encompasses genomic damage accumulation, altered signalling transduction, gene expression to mitogenesis, transformation, mutagenesis and cell death [58] [59]. This may also lead additionally into an accretion of oncogenic mutations that is implicated in cancer pathogenesis and tumor progression. The diagram from Figure 1 explicates the effect of chemical alteration on DNA that involves purine; pyramidines and their hydrogen bonding that subsequently impinge upon DNA replication. Adversely, oxidative impairment to protease and local tissues instigate tumour growth and metastasis.

CONCLUSION

Development of modern methods for the detection of apoptotic cells was the driving force for the discovery of drugs with apoptotic cells death inducing properties for cancer therapy.

CONFLICT OF INTEREST

Authors declare no conflict of interest in the present work.

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