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A Glance at the methods for detection of apoptosis qualitatively and quantitatively

[Apoptozun nitel ve nicel olarak tespitine yönelik metotlara kısa bir bakış]

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ABSTRACT

While programmed cell death, apoptosis, occurs as a necessary and natural event for multicellular organisms, necrosis is a form of unplanned cell death as a result of pathological or chemical trauma. There are numerous molecular and morphological differences between these two forms of cell death, whose decision is based on the type and dose of the stress. As apoptosis is critical for homeostasis of an organism, i.e. for development to adult stage, disease progression, or response to different stimuli, it is being studied more extensively in the area of basic research and clinics, and the need for quick detection of apoptosis and well established criteria for the discrimination of apoptosis have also gained more popularity. Here, we review our knowledge on the most commonly used methods for both qualitatively and quantitatively measuring apoptosis, including morphological imaging (i.e. through light, fluorescence, phase contrast or electron microscopy), immunohistochemical (i.e. Annexin V-FITC, TUNEL, M30 antigen or caspase 3 detection), biochemical (i.e. DNA- or protein-based electrophoresis or flow cytometry-based methods), immunological (i.e. ELISA), and molecular biology techniques (i.e. array-based techniques) while focusing on the differences for distinction between the two forms of cell death. Indeed, one has to confirm that cell death occurs through apoptosis based on more than one of these protocols depending on the specific purpose of the user.

Key Words: Hematoxylin/Giemsa Staining, Propidium Iodide/Hoechst Staining, Annexin V-FITC, TUNEL, M30 Antigen, Caspase-3, DNA fragmentation, Cytochrome *c*

ÖZET

Programlı hücre ölümü olarak da bilinen apoptozis çok hücreli organizmaların homeostazisi için gerekli ve doğal bir süreç olmasına rağmen, nekrozis patolojik veya kimyasal bir travma sonucu meydana gelen plansız bir ölüm türüdür. Hücresel stresin türüne ve dozuna bağlı olarak gerçekleşen bu ölüm türleri arasında birçok moleküler ve morfolojik değişiklikler bulunmaktadır. Organizmanın gelişimi, hastalık oluşumu veya hücre dışı uyarılara cevap olarak gerçekleşen apoptozis mekanizmalarının hem temel hem de klinik bilimlerde daha fazla çalışılmaya başlanmasıyla, hızlı tespit metotlarının geliştirilerek apoptozis ve diğer hücre ölüm türleri arasında ayırım yapılabilmesi önem kazanmıştır. Bu derlemede, apoptozisin nitel ve nicel incelenebilmesi için morfolojik görüntüleme (örn, ışık, floresan, faz kontrast veya electron mikroskopisi), immünohistokimya (örn, Anneksin V-FITC, TUNEL, M30 antijen veya kaspaz 3 deteksiyonu), biyokimya (örn, DNA- veya protein-bazlı elektroforez veya akım sitometrisi-bazlı metotlar), immünoloji (örn, ELISA) ve moleküler biyoloji tekniklerinin (örn, array-bazlı teknikler) nasıl kullanıldığı hakkında günümüzde kullanılan uygulamalar gözden geçirilmiş ve bu metotlar ile apoptozisin nekrozisten nasıl ayrılabileceği üzerinde durulmuştur. Önemli olarak, apoptozis yukarıda bahsedilen en az iki yöntemle tespit edilerek doğrulanmalıdır. Hangi yöntemlerin seçileceği konusunda araştırıcının amacı belirleyici unsur olmalıdır.

Anahtar Kelimeler: Hematoksilen/ Giemsa Boyaması, Propidyum iyodür/Hoechst Boyaması, Anneksin V-FITC, TUNEL, M30 Antijen, Kaspaz-3, DNA fragmentasyonu, Sitokrom *c*

Historical Perspective for Apoptosis

Many methods have been developed in order to detect apoptosis in vitro. When the term "apoptosis" was first used in 1972, the definition was made according to morphology of the cell [1]. However, nowadays apart from cellular appearance, this definition can also be established through determining changes at the molecular level.

The methods to detect apoptosis also developed as the definition of apoptosis developed to include molecular mechanisms during this process. For instance, while morphologic criteria were the main basis for evaluation initially, researchers began to analyze the emergence of DNA breaks towards the end of 1980s and look for active caspases by the mid 1990s with the new discoveries in the apoptosis field [2]. At the end of 1990s, tools to detect translocation of phosphatidiylserine from inside to the outside of the cell were developed to detect apoptosis. During the early 2000s, a novel biomarker called the caspase cleaved cytokeratin 18 came into play and is still being used extensively. This biomarker for apoptosis, that is also called M30 antigen, is only specific for cells that are of epithelial origin. In summary, the advancements in technology have provided better apoptosis assays over time.

Methods for Determination of Apoptosis

Methods used to determine the presence of apoptosis are listed below. It is considered that the apoptosis assays need to not only quantify the extent of cell death, but they must also be able to distinguish between the various pathways [3].

1. Morphology-based methods
2. Immunohistochemistry-based methods
3. Biochemistry-based methods
4. Immunology-based methods
5. Array-based methods

Different kinds of methods given above can be used for the detection of apoptosis-related molecular events. For example, if apoptosis is to be determined on the basis of DNA fragmentation, one or more of the methods listed above may be employed. This is because DNA fragmentation can be demonstrated both histochemically and biochemically, and also by the use of ELISA. The most important aspect of this differentiation is the type of the sample in question. If the sample is a product of cellular growth from a culture, agarose gel electrophoresis, which is a biochemical method, may be used. If DNA fragmentation (apoptotic cells) in a tissue is being investigated, the TUNEL method, an immunohistochemical technique, may then be employed.

There are overlapping features between different forms of cell death; hence one method is not good enough to confirm apoptosis. Therefore, as a critical point in apoptosis research, the confirmation of apoptosis should be made by another method. But this should not be made by the same class of method. For example, if the apoptosis is determined on the basis of morphology of the nucleus, then the confirmation may be based on a biochemistry-based method such as agarose gel electrophoresis for the DNA cleavage.

1. Morphology-based methods:
   - 1.1 Light Microscope
     - a. Hematoxylin Staining
     - b. Giemsa Staining
   - 1.2 Fluorescence Microscope
     - a. Hoechst Dye staining
     - b. Propidium Iodide staining
   - 1.3 Electron Microscope
   - 1.4 Phase Contrast Microscope

1.1 Light Microscopy: Apoptosis was recently reported by Jain *et al* to be fairly accurately assessed using light microscopy after staining the cells [4]. Mainly two dyes are available for the staining and light microscopic evaluation as given in detail below.

a. Hematoxylin Staining: The cheapest and the easiest of the morphological staining methods is that with hematoxylin staining. Materials stained with hematoxylin are visualized with the light microscope, which is available almost in all laboratories. Hematoxylin staining may be used in both cells in vitro [5, 6] and tissues in vivo [7, 8]. It is convenient to start with this method in the determination of apoptotic cells, and it is advantageous over other methods for several reasons (for example, initial evaluation in a fast and cheap way).

As hematoxylin stains chromatin, apoptotic cells are evaluated according to nuclear morphology. Changes that are specific for apoptosis may easily be observed if the staining was well performed. However, experience is needed, since the mitotic cells at the early stages (e.g. prophase or metaphase) may be confused with apoptotic cells in some instances. The following may be observed in the apoptotic cells: cellular shrinkage, chromatin condensation and its accumulation at the periphery of the nuclear membrane, pyknosis or nuclear fragmentation. In figure 1a, the nuclear fragmentation and chromatin condensation can be observed. In untreated cells several mitotic cells are also visible in different stages of the cell cycle (Figure 1b).

b. Giemsa Staining: As it is with hematoxylin staining, determination of apoptotic cells by Giemsa staining is made on the basis of cellular morphology. Although the borders of the cytoplasm are more accurately distinguished with this method compared to hematoxylin staining, there is no significant superiority. This staining is also cheap and fast with less than one hour as total duration.

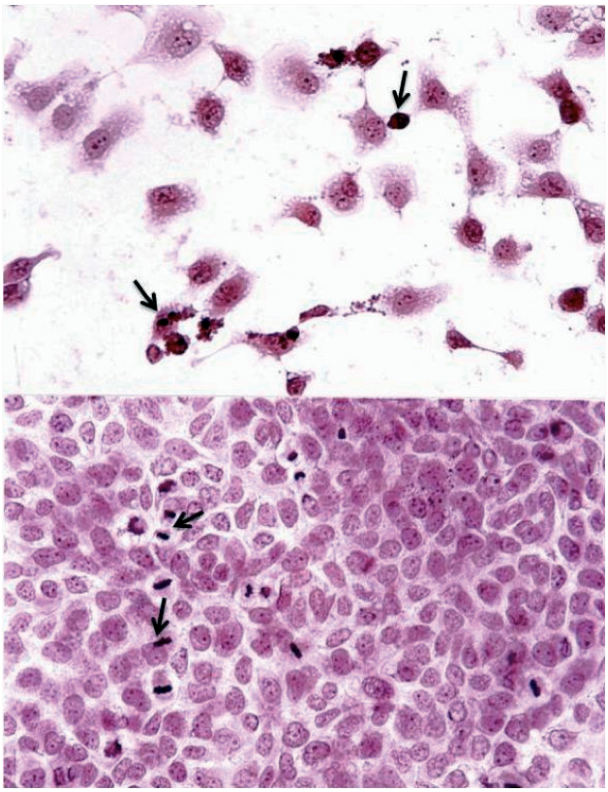

Figure 1. Morphological evaluation of apoptotic cells (A431 epidermoid carcinoma cells) stained with hematoxylin (Magnification 200X). A) Condensed chromatin (darker than the others) and fragmented nucleus are observed in apoptotic cells (top panel indicated by black arrows). Some blebs may also be seen with careful examination. B) Healthy cells (bottom panel,) do not show such morphological changes. The nucleus in the prophase may be confused with the apoptotic figures while those in the metaphase are relatively easier to discriminate. Cells in metaphase are indicated by arrows (bottom panel, B).

2.2 Fluorescence Microscopy: In this method the staining procedure is performed with fluorescent substances like Hoechst dye 33342, DAPI or Propidium Iodide. As florescent dyes are bound to DNA, the chromatin, and as such, the nucleus of the cell becomes visible. Fluorescence systems are considerably more expensive than light microscopy, but if used in cellular culture studies, they may be beneficial in differentiating live cells from dead cells. However, in samples where Giemsa or hematoxylin staining is used, all cells are observed dead due to the fixation of cells. In order to differentiate live and dead cells, a substance that dyes both live and dead cells (for example, Hoechst dye 43332) is combined with another substance that dyes only dead cells (for example, propidium iodide). The basic principle of this method in identifying viability is to determine whether the plasma cellular membrane is intact or not. Cells with intact cellular membranes (live cells) are not stained with a dye like propidium iodide which only stains cells with defective membranes (dead cells). Hence cells may be double stained with a substance like the Hoechst dye that stains all cells regardless of their viability providing the chance to differentiate dead cells from living ones (Figure 2a). In addition, whether or not dead cells die due to apoptosis or necrosis is investigated by examining the nuclear morphology, as it is the case with hematoxylin staining. Therefore, flourescence imaging allows discrimination between apoptotic and necrotic cells as well.

Cells with chromatin condensation or nuclear fragmentation suggest apoptosis (Figure 2b).

A further definition of cell death is made using the following criteria:

- Cells that die through necrosis: There are no apoptotic changes in the nuclei of cells whic h are identified as dead. Both propidium iodide and Hoechst dye stain the nucleus, which gives purple-like color. There is no significant change in the nuclear pattern. But the nucleus of a necrotic cell may be observed larger than normal. The intensity of the dye may be normal at the beginning, but it may be weaker in later stages due to the lysis of the cell.
- Cells that die through apoptosis: If there is no secondary necrosis (that happens in the late stage), the cellular membrane is always intact. Hence, the apoptotic cells at the early stage are not stained with propidium iodide, but the Hoechst dye is positive. However, the diagnostic finding in these cells is the nuclear morphology, which should show some specific appearances for apoptosis as explained above. Therefore, typical nuclear fragmentation or the chromatin condensation is the most important feature for apoptotic cell death.
- Normal (live) cells: Propidium iodide is negative. They are positively stained with Hoechst dye and their nuclei look normal.

As a summary, in necrotic cells, the Hoechst dye is (+) and propidium iodide is (+); in apoptotic cells, Hoechst dye is (+), propidium iodide is (-) and apoptotic morphology is (+); in normal cells, Hoechst dye is (+), propidium iodide is (-) and apoptotic morphology is (-). In addition to the nucleus dyes, some fluorescent probes (*e.g.* a green probe FLIVO) are also available to detect apoptosis via caspase activation [9].

2.3 Electron microscopy: In apoptosis, evaluation with the electron microscope is considered as the most valuable method ("gold standard") [10]. It is a method where morphological changes are best observed. Moreover, subcellular details like, condition of the mitochondrium, whether or not the integrity of cellular or nuclear membrane is preserved, may be investigated.

Figure 3 is an example of electron microscopic evaluation: the fragmentation of the nucleus can clearly be observed. The cellular membrane looks intact and the chromatin seems condensed and aggregated at the periphery of the nucleus.

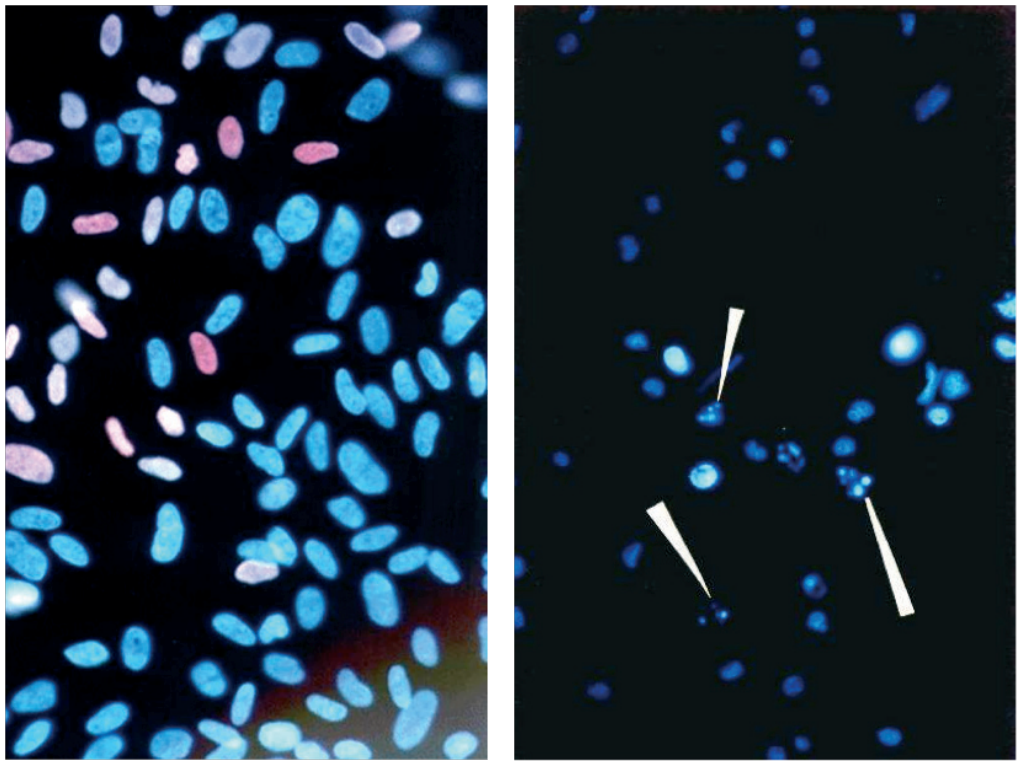

Figure 2. A) Double (Hoechst 33342 Dye and Propidium Iodide) staining of A431 (epidermoid carcinoma cell) line (Left panel, Magnification 400X). Some nuclei stain in reddish while the others stain in blue only. Because the reddish-stained cells are those which lost the intactness of their cell membrane, propidium iodidegets into the cell and stain the nuclei in red (necrosis). B) The nuclear fragmentation is clearly seen in apoptotic cells stained with Hoechst 33342 dye (indicated by the arrowheads). (Magnification 200X).

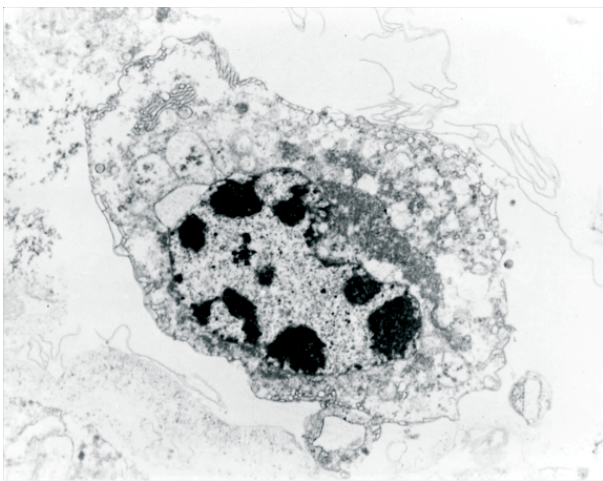

Figure 3. Electron microscopic examination of apoptotic cells (A431 epidermoid carcinoma cells). The nucleus is fragmented. The chromatin is condensed and aggregated at the periphery of the nuclear membrane. The cell membrane is still intact. Some vacoules are also observed in the cytoplasm.

2.4 Phase Contrast Microscopy: This type of a microscope can be used in order to investigate cells grown up in cell-culture flasks or plates. As dead cells detach from the substratum where they are bound, they begin to float in the culture medium. These floating cells can easily be identified with the phase contrast microscope. Cells undergoing mitosis may also be determined using the phase contrast microscope, but they may be confused with the very early-stage apoptotic cells, because they have spherical appearance like the early stage apoptotic cells. Therefore, it may not be easy to differentiate these two distinctive cellular events. During both mitosis and the earlier stages of apoptosis, the cells appear spherical and smaller, but are not spread on substratum to which they are attached. Blebs may be seen on apoptotic cells with careful examination by the phase contrast microscope (Figure 4). If the cells are still spread on substratum, vacuoles that develop in the cytoplasm may also be seen. Moreover, in some cases, the blisters, which run over from the cytoplasm may also be observed. These blisters may get detached from the cell and begin to swim in culture medium. Their interior is seen as empty spherical structures. These detached blisters are probably "the ghost cells" as defined in the literature. Although the membranes of the cells that proceed to apoptosis are intact at the beginning, secondary necrosis develops during the advanced stages and then the integrity of their membranes is thus impaired. If non-vital dyes like propidium iodide are administered to these cells before secondary necrosis, they are not stained despite the fact that apoptosis is already present, because their membranes are intact. As the integrity of the membrane is impaired following secondary necrosis, these cells get stained with non-vital dyes.

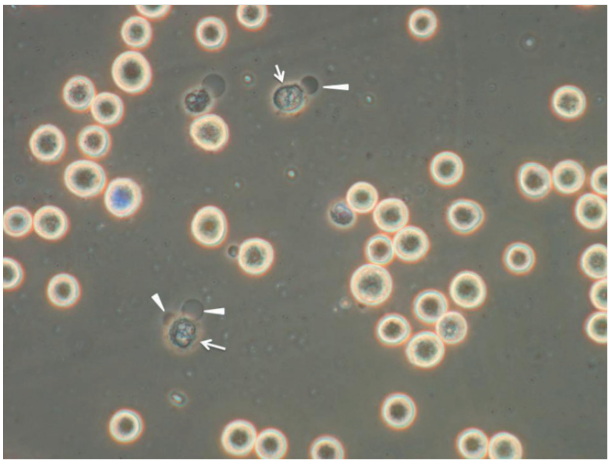

Figure 4. Phase contrast imaging of cells (A549, non-small cell lung cancer cells): Most of the cells are healthy with circular appearance. A few of them, which seem to be apoptotic with this examination, do not have a clearly-visible cell membrane (indicated by arrows). In addition, those cells have one or two blisters (indicated by arrowheads, ghost-like appearance). Those cells do not stain with propidium iodide at this stage, which means that the cell membrane is still intact. (Magnification 200X).

At the blister stage, the integrity of the membrane is still intact and the changes of nuclear morphology may be observed with florescent dyes. However this stage does not last long and the integrity of the membrane becomes impaired within minutes. Normally no dye is needed in order to observe cells with the phase contrast microscope, however as mentioned above the phase contrast microscope may be used with florescent dyes as well at the same time. By doing so, the phase contrast images and the fluorescent images overlap and gives detailed information of both nucleus and the cell membrane.

2 Immunohistochemistry based Methods:

2.1 Annexin V-FITC Assay

2.2 TUNEL Method

2.3 M30 Antigen Detection

2.4 Active Caspase-3 Detection

2.1 Annexin V-FITC Assay

One of the membrane phospholipids, phosphatidylserine (PS), is normally located in the cytoplasmic surface of the membrane. Once the cell undergoes apoptosis, the PS molecules are translocated to the outer surface of the cell. This translocation allows determination of PS, and in turn apoptosis, when an agent such as Annexin-V labeled with a florescent substance (for example, FITC) is applied to the cell environment [11,12]. And then, using a fluorescence microscopy allows to detect apoptotic cells. However, one must be careful in the interpretation of data, as a cell undergoing necrosis could also be stained with annexin V+FITC, since the label may enter in the cytoplasm through the damaged membrane and stain the cytoplasm-exposed PS molecules, giving a false positive result. If a fluorescence microcopy is not available, then it is also possible to use annexin V-FITC in a flow cytometry-based method [13].

2.2 The TUNEL Method

TUNEL (**T**dT-mediated d**U**TP-biotin **n**ick **e**nd-**l**abeling) provides determination of DNA breaks in situ [14]. Paraffin blocks, frozen sections, or cultured cells can easily be analyzed by this method. This assay gives clear images of apoptotic cell death in a population of cells (Figure 5). In general, the immunohistochemistry rules are applied to the samples. But, instead of proteins, the DNA is under study in this assay. The nuclei may be visualized by either colorimetric or flourometric methods. The apoptotic cells give fragmented and/or condensed appearance (or both) with positive staining in brown if DAB (a colorimetric dye) is used. The normal cells are stained in green with methylene green or red-bluish with hematoxylin. Figure 5 represents an example of methylene green staining where normal cells are colored in green and apoptotic cells stained in brown. However, there are some limitations of the TUNEL assay. Some of the nuclei labelled by the TUNEL assay may, in fact, be transcriptionally active (proliferating cells). In addition, the duration of proteolytic treatment and TdT concentration are factors that have been found to greatly influence results [15,16]. Therefore, it is best to approach TUNEL assay with caution.

2.3 M30 Antigen Detection

In this method, apoptotic cells are determined via appearance of a novel epitope of cytokeratin 18 as a result of breakdown through caspase activation. This new epitope is visualized by using a specific antibody targeting this cleavage site [17,18]. This method can only be used in tissues that express cytokeratin 18, which are of epithelial origin [18]. Therefore, the apoptosis in hematological cells cannot be determined. In placental tissues, M30 antigen detection seems to be useful to detect apoptosis [19]. The basic immunohistochemical rules are applied in this assay.

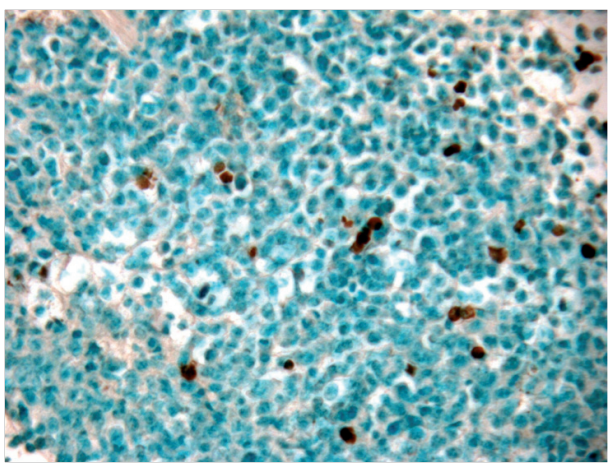

Figure 5: TUNEL staining of Ehrlich ascites tumor cells grown in Balb/c mice. Paclitaxel was used to induce apoptosis. The TUNEL positive cells are those which are stained in dark brown in both nucleus and cytoplasm. Cytoplasm could also be stained due to the leakage of cleaved DNA into the cytoplasm although it is not always necessary to observe. Background staining was performed with methyl green. (Magnification 100X).

2.4 Active Caspase-3 Detection

Procaspase-3 is a protease playing a key role in apoptosis in various different cell types [20]. Procaspase 3 is activated by different stimuli inducing apoptosis and then it is called cleaved or activated caspase 3. Both zymogen (uncleaved) or cleaved form of caspase 3 can be detected immunohistochemically. The detection of the cleaved form of caspase 3 provides a method that is specific for the determination of apoptosis in the cells / tissues. However, one has to know in advance that whether or not caspase-3 is expressed in the cell / tissue of interest. For example, the cerebellum of mouse and plasental tissue of baboon seem to be a good models to detect active caspase-3 immunohistochemically [21, 22].

3 **Biochemistry-Based Methods:** Biochemical methods are particularly important in the clinics, mainly due to their ease of performance and also for the better management of patient care (e.g. cancer cases) through determination of apoptosis; albeit not yet routinely applied. Among the potential biomarkers of apoptosis, cytokeratins and DNA nucleosomes [23] are especially of importance. Both of them are easily measured by ELISA, which is a common technique in most of the routine clinical chemistry laboratories. Furthermore, plasma total cytokeratin 18 level was reported to be a strong prognostic marker in non-small cell lung cancer patients (CI: 0.50-0.82, p=0004) [24].

3.1 Agarose Gel Electrophoresis-DNA fragmentation

3.2 SDS-PAGE and Western Blotting

- **a.** Substrate Breakdown
- **b.** Determination of Active Caspase 3
- **c.** Release of Cytochrome *c*

3.1 Flow Cytometry

- **a.** DNA reduction
- **b.** Annexin V-FITC

3.1 Agarose Gel Electrophoresis:

During apoptosis, the genomic DNA is chopped into ~180 base pairs or its multiples at certain regions (internucleosomal regions), hence appears as a ladder when electrophoresed (Figure 6) [25]. For this reason, this finding is a characteristic, hallmark feature of apoptosis and is not present in necrosis. (In necrosis the cells give a smear pattern on the gel.) As a result it is one of the methods used to differentiate apoptosis from necrosis. In fact, in one recent study, the agarose gel electrophoresis along with the other methods (e.g. fluorescence microscopy and caspase-3 activity) was successfully used to confirm apoptosis and necrosis in one recent study [26].

3.2 Western Blotting

This method can be used for various purposes such as determining whether certain proteins that are specific for apoptosis are expressed or not (for example, M30 antigen) or whether or not they are broken (e.g. PARP). Alternatively, it can be used to determine in which part of the cell a specific molecule is located, such as cytochrome c, which is released from mitochondria into the cytoplasm during apoptosis [27]. To use western blotting for detection of cytochrome c release, a clean fractionation should be performed in order to differentiate mitochondrial from cytoplasmic fractions of cells. Consequently, detection of cytochrome c in cytoplasmic fraction is an indication of apoptosis. But, a risk of contamination of cytoplasmic fraction with mitochondrial fraction is always possible. Therefore, a mitochondrial marker, called cytochrome c oxidase, should also be employed for the detection of such contamination (Figure 7). In the case of the presence of this marker in the cytoplasmic fraction, the experiment should not be considered valid.

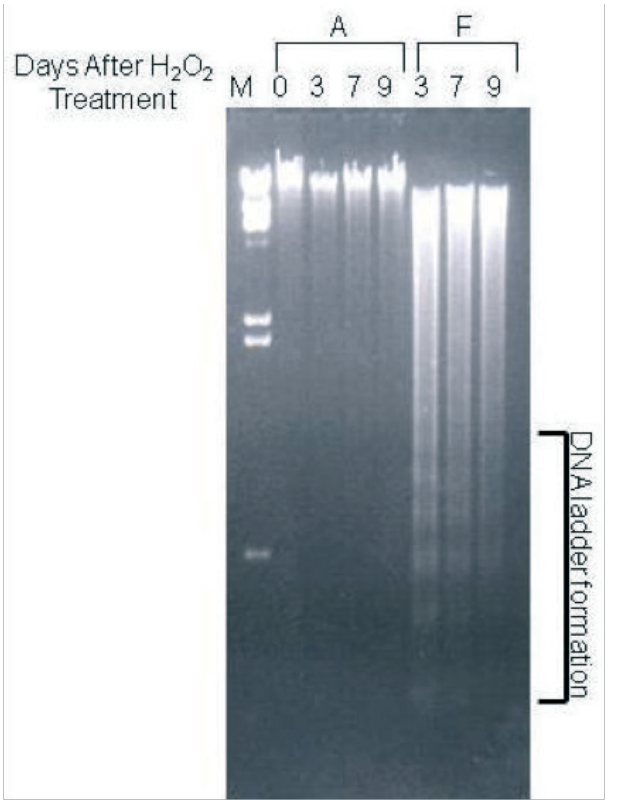

Figure 6: Oral squamous cancer cells are treated with 4mM H_2O_2 and lysed at indicated times after treatment. The genomic DNA is isolated and electrophoresed on agarose gels. Apoptotic DNA ladder formation is observed mostly in floating cells. (A: Cells that are Attached on the plate, F: Cells that float)

3.3 Flow Cytometry

Any surface protein, whose expression is known to change during apoptosis may be determined with flow cytometry, through the use of an antibody labeled florescent substance, proving a useful tool for the determination of apoptosis [13]. This method is particularly useful in clinics for the detection of apoptosis, due to its

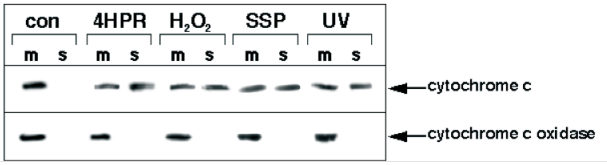

Figure 7: Determination of cytochrom c release from mitochondria (labeled m) to cytoplasm (labeled s) in cells undergoing apoptosis that is resulted from various stimuli. Con: untreated control cells (A431 epidermoid carcinoma cell line), 4HPR:fenretinide, SSP: staurosporine, UV: ultraviolet. (Adapted and reprinted by permission from *Cell Death Differ* (39)).

ease in application, ability to deliver quantitative results and less time requirements. Detection of apoptosis may be performed in two ways: 1- through the use of propidium iodide, a fluorescent substance; and 2- through the use of Annexin V labelled with FITC (Fluorescein isothiocyanate). In the first way; apoptotic cells have a decreased propidium iodide fluorescence and diminished forward light scatter compared with the cells in the main peak (G1). Therefore, the collection of apoptotic cells is detectable at sub-G1 peak as shown in Figure 8. In the second way; the annexin V binds to the phosphatidylserine which becomes exposed on the outside of the plasma membrane during apoptosis. Because FITC (a fluorescent dye) is attached to the annexin V, it is possible to detect the apoptotic cells by a flow cytometry. In this method, the other fluorescent dye, PI, may also be used to detect necrotic cells concomittantly. In the annexin V-FITC method, a fluorescence microscopy can be used to detect the apoptotic cells but flow cytometry can of course provide a much faster analyses.

4 Immunology-Based Methods:

4.1 ELISA

- **a.** DNA Fragmentation
- **b.** Caspase-cleaved cytokeratin 18 (M30 antigen) Level

4.1 Fluorimetric Method

Active caspase 3

4.1 ELISA

It is possible to detect internucleosomal DNA fragmentation by ELISA both in cell culture and human serum/plasma. In addition to DNA fragmentation, caspase-cleaved cytokeratin 18, also named M30 antigen, which is a novel biomarker for apoptosis, in either cell culture or plasma/serum is measured by ELISA (Apoptosense ELISA assay, Peviva, Sweden). The detection of M30 antigen is particularly convenient and reliable to measure apoptosis quantitatively in any suitable human sample (Figure 9). M30 antigen detection by ELISA is reported to be useful in the management of cancer patients [28-32] and as a diagnostic tool for one of the common liver diseases (NASH) [33]. Figure 9 shows the effect of cisplatin and paclitaxel in the induction of apoptosis

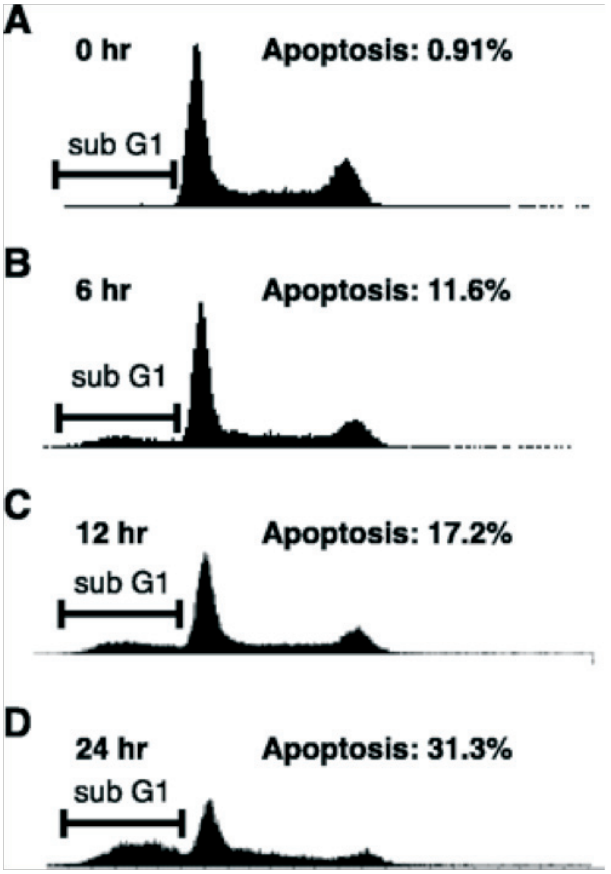

Figure 8: Flow cytometry analysis of apoptosis in DLD-1.ApoL6 cells by propidium iodide staining. DLD-1.ApoL6.V5 cells were grown to 50% confluence in D.20 medium and then switched to D.0 medium. Cells were harvested hours after induction: (A) 0 hour, (B) 6 hours, (C) 12 hours, and (D) 24 hours (Adapted and reprinted by permission from the *American Association for Cancer Research*: (40)) .

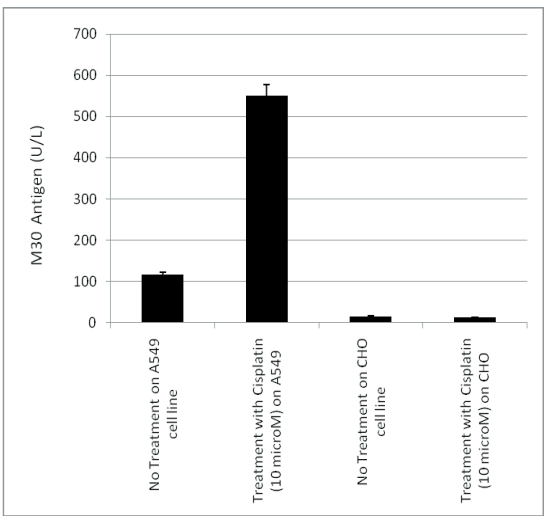

Figure 9: M30 antigen detection by ELISA (Apoptosense ELISA Kit, Peviva AB, Sweden). This assay detects only the human-originated M30 antigen. A549 cells originate from a non-small cell lung cancer, while CHO cells are established from Chinese Hamster Ovaries. CHO cells are used as negative control for the determination of M30 antigen level, since M30 antigen is not available in this cell line due to the animal origin of CHO cells.

by detecting the caspase-cleaved cytokeratin 18 (M30 antigen). It shows that M30 antigen increases in A549 non-small cell lung cancer cell line by treatment with cisplatin (10 μM) and paclitaxel (7 μM) for 48 hours. CHO (Chinese Hamster Ovary) cell line is used for the negative control in which there is no cytokeratin 18 that is detectable by the M30 antigen ELISA assay in this cell line. In fact, M30 antigen assay is not used for the detection of apoptosis in rodents [34]. But, M30 antigen assay is particularly useful for determination of drug-induced tumor growth inhibition via apoptosis in animal studies using human xenograft tumors [34,35]. However, one should take into account that acute tumor cell death is not the sole determination of response to the anti-cancer drugs [36].

4.2 Fluorimetric method

This method is used to determine the activities of caspases (e.g. caspase-3 or caspase-9) in cultured cells [37]. First, active caspase molecules in the cell lysate are captured by relevant antibodies to the microtiter plates. Then a substrate is added to the milieu that is broken by captured caspases. The intensity of fluorescence depends on the activity of caspase in the sample.

5 Array-Based Methods:

5.1 DNA Microarrays

Gene Expressions (mRNA)

DNA microarray technology is a new and relatively expensive method. However, because one can detect many apoptosis-related genes in the same run, it may also be regarded as a cost-effective method. In fact, detection of hundreds of gene expression (mRNA) in one run may be possible to analyze either apoptosis- and cytotoxicity/genotoxicity-related pathways [38]. As apoptosis takes a tremendously important place in medicine, it may be expected that the detection of apoptosis-relevant genes by array-based technologies can change medical practice radically in the future. As such it will especially be possible to obtain extensive information about expressions of cell surface death receptors that are specific for apoptosis. Currently, some companies have already introduced new and efficient arrays for the detection apoptosis-related genes. For example, 84 gene / gene parts are possible to analyze in one run using an ordinary 96-well cell culture plate by RT-PCR.

Conflict of interest:

The authors declare that there is no conflict of interest.

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