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Efficacy of Nuclear Area Factor (NAF) and Nuclear Morphology Changes in Assessment of Apoptosis

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Abstract

Aim of the study: *To evaluate the importance of nuclear morphological changes and nuclear area factor as an early indicator of cellular morphological changes occurring during apoptosis.*

Background: *Apoptosis is considered as tightly programmed cell death with characteristic genetic and biochemical pathways that have an important role in the growth, development and homeostasis of normal tissues. It leads to the removal of unnecessary and unwanted cells to retain the healthy balance between cell death and cell survival. Through apoptosis, apoptotic cells undergo distinctive morphology. Typically, after cleavage of laminin and actin filaments in the cytoskeleton, the cell begins to shrink followed by a breakdown of nuclear chromatin leads to nuclear condensation and a "horseshoe" like appearance. Caspase-3 expression in apoptotic cells showed the highest correlation with a morphologic indicator defined as the nuclear area divided by a form factor called nuclear area factor (NAF).*

Keywords*: Apoptosis, Nuclear area factor, Image analysis, Caspase-3.*

Introduction

Apoptosis represents physiological cell death and the active process of self- tissue destruction that occurs during growth and regulation of tissue homeostasis or due to changes in environmental stimuli **(**1**)**. Apoptosis induction is resulting from the absence of specific reactions between extracellular matrix proteins and integrin transmembrane receptors **(**2**)**. Stimulation of caspases enzymes is necessary for apoptosis activation **(**3**)**, can be as inactive form with cellular organelles or with signal transduction complexes **(**1**)**.

In many experiments involving cell cultures, Cell apoptosis rate is considered an important parameter. Apoptosis kinetics can be evaluated by counting the total number of cells and area occupied on each culture dish through analyzing images taken at different times of their evolution, apoptotic cell death resulting in condensation and fragmentation of cell bodies, forming areas filled with unstructured smaller objects **(**4**)**.

Apoptosis passes through typical alterations of nuclear morphology **(**5**)** characterized by a progressive condensation of chromatin that stimulates nuclei to collapse into little single balls or clusters of grapes **(**6**)**. Thus alterations in nuclear morphology can be demonstrated using a nuclear stain such as heamatoxylin **(**1**)**.

The first stage of apoptosis is compatible with an initial stage of activation of caspase-3 that is found in the cytoplasm, characterized by normal nuclear morphology faintly stained by heamatoxylin. The second apoptotic stage, characterized by cytoplasm and nuclear over-staining, may represent the presence of caspase-3 in the nucleus and increase its activation in the cytoplasm. The third apoptotic stage is compatible well with the typical picture of the late phase of apoptotic cell death, characterized by fragmentation of nuclear envelope **(**7**)**.

Based on morphology, cell death can be categorized as apoptosis necrosis or both. In apoptotic cell death, the nucleus undergoes early degeneration while the organelles and cell membrane conserve for some time. In necrosis, the cell membrane and organelles undergo early degeneration whereas the nucleus stays relatively intact **(**8**)**. Typically, one of the first signs of apoptosis is cap-shaped chromatin margination **(**9, 10**)**. Cell membrane blebbing, cytosol condensation and pyknosis can also be noted. Several electron-dense micronuclei develop from the nucleus, which are often moved then into the extracellular space. Finally, the cells divide into numerous apoptotic bodies. Apoptotic cells in vitro undergo a late phase of necrosis accompanied by early cell membrane damage **(**11, 12**)**. However, fragmented and pyknotic cell nuclei are not common in necrotic cell death. Microscopic examination of the cells is considered the primary method for studying cellular function. Biological mechanisms can be revealed by visual analysis when cells are stained appropriately **(**13**)**. In advanced stages of apoptosis, Cells show morphological alterations characterized by cytoplasmic and nuclear condensation and fragmentation of cells into numerous membrane-bound apoptotic bodies. These morphological

alterations are accompanied initially with large and subsequently, very small chromosomal DNA fragments and can be examined by light microscopy **(**14**)**. However, other morphological changes of apoptosis occur without detectable DNA fragmentation or with a decrease in DNA content **(**15**)**.

Morphological features of apoptosis can be used as indicators of activation of programmed cell death due to the characteristic changes in nuclear morphology during apoptotic cell death. A low nuclear area factor (NAF), which was first defined as the nuclear area multiplied by roundness **(**16**)**, has been considered as an early sign of apoptotic cell death**(**16,17,18**)**.

Still, in most applications, automated cell image analysis (image cytometry) is strongly preferred over eye analysis. Sometimes, image cytometry is preferred to extract the full spectrum of information present in biological images due to several causes as it produces several informative measures of cells, measures each rather than producing a score for the entire image, meticulous consistent quantitative measures for every image and in the last, higher-throughput and less labor-intensive **(**13**)**.

Recently, many systems for image analysis in nuclear morphometry are available to an anatomic pathologist. Many of these systems, however, require hardware attachments for image acquisition, expensive software, analysis and storage. So, a cost-effective alternative for image analysis is considered a welcome tool for researchers and pathologists alike. In this context, "Image J" is an analysis program developed at the National Institutes of Health (NIH) and freely available java-based public-domain image processing. Recently, "NIH Image" Image J's Macintosh platform counterpart can be used widely in biologic research. Stained sections can be used to evaluate several nuclear morphometric descriptions after downloading specific plug-ins from the Image J website. The macros and plug-ins can be used as source files and downloaded to the Image J folder **(**19**)**.

Image-Pro Plus software is employed by some studies to allocate scores based on NAF character; a completely round cell nucleus take scores 1 while fewer round nuclei take score above 1. Image J software does not calculate nuclear roundness, instead computes form factor (in Image J called 'circularity). Based on the form factor, a complete circle takes scores 1 and fewer circular structures take scores between 0 and 1. So, to calculate NAF using Image J, the nuclear area can be divided by its circularity **(**17**)**. But, some studies have calculated NAF by multiplying the nuclear area by form factor **(**18, 20**)**.

Changes in chromatin organization and in amounts of DNA during apoptosis can be revealed by image analysis of stained nuclei. This analysis allows the determination of the early stages of apoptotic cell death by correlating slight alteration in nuclear morphology with a decrease in the amount of DNA **(**21**)**.

The number of biochemical and image-based essays for apoptotic cell death are available that vary greatly in complexity, cost and specificity **(**22**)**. It has previously revealed that NAF can be used as an early indicator of morphological changes of cells occurring during apoptotic cell death **(**23**)**. Therefore,

NAF calculation is relatively straightforward and can be determined by a number of image analysis programs, NAF calculation remains a valid marker of changes in apoptotic, as long as the nucleus is intact and the DNA doesn't completely damage, even though the greatest extent of these changes occur early stage of apoptosis**(**22**)**.

By the image analysis method, variation is produced through systematic undercount of nuclei because of the inability to properly determine the count of the nucleus when two nuclei touch one another. So, thin sections are needed by the image analysis method to decrease this problem **(**24**)**.

It was a disparagement for image analysis software program Image J 1.37 v, when Image J was free available, all of the pre analysis filters and features weren't included that is needed for separating cells before calculating NAF or other measurements especially in cases where cells may aggregate in vitro either normally or under the apoptotic stimulus, and tools used in Image Proplus for splitting objects are useful for calculating NAF **(**22**)**.

NAF calculation could be used easily with nuclear dye as Giemsa or hematoxylin, the advantage of using these blue dyes is that they allow for simultaneous staining of the same cells with independent markers for apoptosis such as FITC-based TUNEL, NAF calculation performed as the result of area (in pixel2) roundness. Using image J data for calculating NAF, when roundness was not an available function, circularity was used as an alternative **(**22**)**.

The existence of a variety of apoptosis inducers and of variable expression according to the cell type considered has led to a reconsideration of the morphological criteria for the detection of apoptosis. Even in studies using in situ labeling of DNA strand breaks as a marker, there is a heavy reliance on morphological features for the identification of apoptotic nuclei. The efficacy of Image J 1.43 n as open-source software in the assessment of apoptosis is a complex and multistep process.

Image J is software that is freely available on the web but does not allow particle analysis of colored images. So, images were converted to 8-bit grayscale, then thresholded. Using the thresholded images, each object was outlined and numbered. Another function of Image J is "cleaning edges", where cells on the edge of the image were removed by the image border and excluded from the final analysis. Image J old version did not have a "split" function to separate closely touching cells. So the thresholding cannot be separate thus cells and remain as large collections, also need to be excluded from the final analysis, but this disadvantage was improved in the current version. The current versions of Image J possess the watershed function where they can split the closely touching cells so cells can be split by the watershed function but cannot be separated by thresholding. Other functions of the new version of Image J are cell counting added to results in more objects that can be included in the count. While in the old version of Image J, objects can be theoretically separated using thresholding, as it was a damaged process, that makes all objects smaller when thresholding is increased, the option of automatic thresholding is available in the new version using a sample of the desired color so thresholding is more accurate.

Therefore, both ways of NAF calculation have been greatly associated with apoptotic cell death, which of these two definitions of NAF is optimal has not been revealed. As well as, these studies did not compare the nuclear morphology with apoptosis-related proteins expression in each cell; instead compare average nuclear measurements in apoptosis-induced cultures with healthy control cultures. The sensitive method to identify morphological characteristics is comparing individual cell morphology with apoptotic phenotype, as apoptosis has an affect on cellular morphology, the relationship between the cells may be changed in cultures containing a higher number of apoptotic cells. By using nearest analysis, the distance between each individual cell to its nearest neighboring cell is measured to calculate an R-value, R-value of 0 means a completely clustered growth pattern, while R-value of 1 denotes cultures with completely random spacing between cells. Considering the theoretical scenario of even spacing between cells, the R-value denotes 2.15 when cells are distributed in a hexagonal pattern. While using the nearest neighbor analysis to evaluate whether apoptotic cell cultures demonstrate specific cell distribution patterns have not been undertaken **(**25**)**.

Regarding, the relationship between objective changes in nuclear morphology and cellular distribution following caspase-3 induction in

apoptotic cells, it is shown that morphological changes accompanied with caspase-3 activation could be objectively quantified. A novel morphological indicator, defined as nuclear circumference divided by form factor, showed the strongest association with caspase-3 expression. For example, nuclear morphometric measures of mean nuclear elongation factor, mean nuclear regularity factor and mean nuclear area were considered as potential predictors for clinical outcomes in localized renal carcinoma **(**26**)**. Furthermore, regarding prognostic factors in prostate cancer, it was reported that factors such as nuclear area and nuclear circularity required further studies before routine use could be recommended **(**27**)**.

Shortly, NIH Image/Image J gives a lot in spite of being freeware; knowledge of its potential uses will produce reproducible, objective, timesaving and cost-effective methods of automated image-based IHC evaluation and morphometric analysis for histopathologists.

Conclusion

We conclude that caspase-3 positive apoptotic cells showed morphological changes that can be quantified objectively using freely available Image J software. A novel morphological indicator, defined as nuclear circumference divided by form factor, showed the strongest relation with caspase-3 activation. Comparison of individual cellular phenotype and nuclear morphology using Image J can potentially lead to the development of defined morphological indicators of activation of specific cellular pathways.

Nuclear area factor can be calculated using powerful free and open-source software; NAF estimation is a sensitive indicator of the early apoptotic effect of anti-cancer therapy. So, a quantitative measure of apoptosis can be obtained that is correlated to morphological features.

Therefore, Image J 1.43 n may provide an important tool for the discrimination and assessment of apoptotic cells.

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