Methods of apoptosis detection

Metody wykrywania apoptozy

Agnieszka Pedrycz[1], Piotr Siermontowski[2], Marcin Orłowski[3]

[1]Department of Histology and Embryology Medical University in Lublin
[2]Department of Maritime & Hyperbaric, Medicine Military Institute of Medicine, Gdynia
[3]Centrum of Diagnosis and Therapy of Digestive System's Diseases Gdynia

Abstract

The aim of present study was analyze of methods of apoptosis detection. We described methods detecting single apoptotic cells and their populations. In those methods we showed: single cell gel electrophoresis (SCGE), apoptotic cell identification using transmission electron microscopy (TEM) and light microscopy; method based on assessment of changes in the apoptotic cell membrane; method based on Caspase 3 activity examination. The other methods exanimate releasing of cytochrome C from the mitochondria and detecting of DNA fragmentation. We described as well flow cytometry (flow cytophotometry). The manuscript was the most focused on immunohistochemical method.

Keywords: apoptosis, methods of detection

Streszczenie

Celem niniejszej pracy była analiza metod wykrywających apoptozę. Opisaliśmy metody wykrywające zarówno pojedyncze komórki apoptotyczne jak i populacje takich komórek. Wśród metod pokazaliśmy metodę kometową, identyfikacje komórek przy użyciu mikroskopu transmisyjnego elektronowego i mikroskopu świetlnego; metodę opartą na ocenie zmian w błonie komórkowej komórki apoptotycznej; metodę opartą na ocenie aktywności kaspazy 3. Przeanalizowaliśmy metody wykrywające fragmentację DNA. Opisaliśmy cytometrię przepływową. Artykuł największą uwagę zwraca na metodę immunohistochemiczną

Słowa kluczowe: apoptoza, metody wykrywania

Introduction

The apoptosis of cells is the suicidal death determining the life of a whole multicellular organism. This is the mechanism naturally balancing the cell proliferation. The healthy cell constantly receives life signals. If the cell does not receive life signals it stops to serve its function and activates its internal programme of suicidal death.

We can distinguish two main pathways leading to cell apoptosis [1]. Extrinsic pathway is induced by an external signal and is connected with the membranous death receptors. In Intrinsic pathway the mitochondria and endoplasmic reticulum are involved.

During apoptotic process in dying cells some proteins (caspases) are activated. The effector caspases destroy nuclear DNA and proteins of the cellular cytoskeleton, including actin, which results in the characteristic changes in the apoptotic cell morphology [2]. Those changes are helpful in detection of apoptotical cells.

Many methods were elaborated to determine the type and course of cell death.

The aim of this study was to describe the most common methods.

Material and methods

Material to this manuscript originated from literature of polish and international medical, histological, biological and chemical journals and books.

We made photographical documentation of immunohistochemical method (standard three steps method), detected caspase 3 (effector caspase of apoptosis) in the liver's and kidney' cells of rats.

We showed as well photographs of morphology of apoptotic cells in the rats' kidney and liver made using light and electron microscopy.

The specimens collected for histological examination in light microscopy from kidney and liver were fixed in 10% formalin buffered to pH 7.4 (phosphatic buffer), dehydrated in a graded ethanol (40%, 50%, 60%, 70%, 80%, 90%, 99.9%), subjected to xylene and then embedded in paraffin.

The paraffin blocks were cut using the 5μm microtone and placed on the slides; after the removal of paraffin they were stained with haematoxylin and eosin.

The epithelial cells of renal convoluted tubules and hepatocytes were analysed. Special attention was paid to

the features suggesting apoptosis. The photographic documentation was prepared using the Jenaval Contrast Carl Zeiss camera. The results were presented in the descriptive form.

The kidney and liver specimens collected for electron microscopy were fixed in the agent consisting of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M phosphatic buffer according to Sorensen [3] and in osmium tetroxide (OsO_4); contrasted in uranyl acetate; dehydrated in the alcohol-acetone series and embedded in the Araldit AMC Fluka resin.

The semi-thin - 0.5-0.7μm and ultrathin - 60nm sections were prepared.

The semi-thin sections were stained with 1% methylene blue with Azure II in 1% water solution of sodium tetraborate, evaluated under the light microscope and photographed using the Jenaval Contrast Carl Zeiss camera.

The ultrathin sections were stained with 8% solution of uranyl acetate in 0.5% acetic acid and lead citrate according to Raynolds. The material was evaluated under the electron microscope Tesla BS-500.

Results and discussion

The methods detecting single apoptotic cells:

Single cell gel electrophoresis (SCGE) [4]. This method is based on the assessment of DNA degradation (unlike in necrosis, DNA in the apoptotic cells is cleaved into fragments of 180 pairs of bases or their multiple),

Next method -apoptotic cell identification using transmission electron microscopy (TEM). The method identifies the morphology of apoptotic cells (Fig.1).

Fig. 1. The damaged tubule with apoptotic cells is present surrounded by the basilar membrane (BM) with normal tubular epithelial cells with normal peribasal striation (double arrows). The apoptotic cells (apoptotic bodies) are visible which contain pyknotic nuclei (N), damaged mitochondria (M) and abundant peroxysomes (arrow). No boundary between dying cells. TEM. Magnification x 3000.

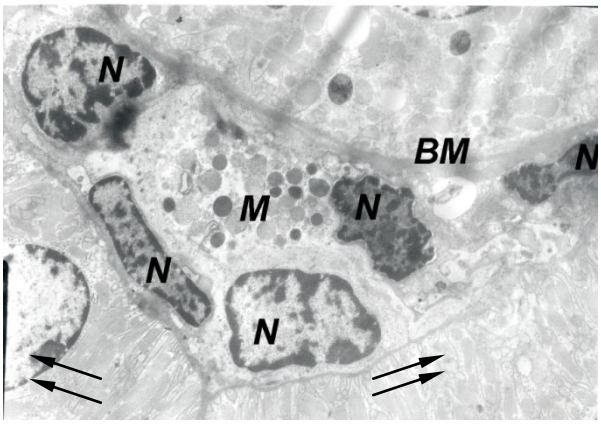


Fig. 2. The liver section of the rat treated with adriamycin and decapitated 4 weeks later. The photomicrograph shows focal abnormalities in architectonics, irregular hepatocytes, hepatocytes with brightened cytoplasm, nuclei of various shape, colour and size, numerous erythrocytes in sinuses. H+E staining. Magnification x 400.

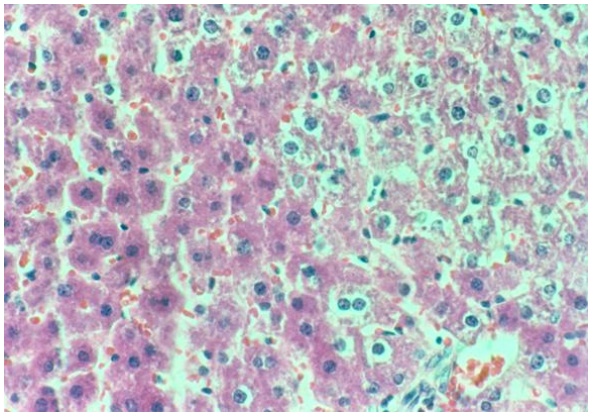
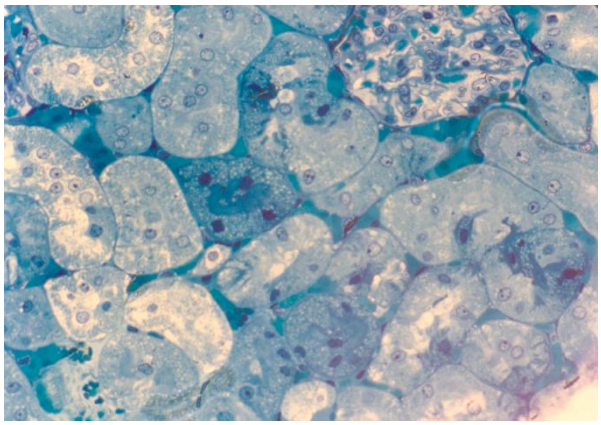


Fig. 3. The kidney section of the rat decapitated 4 weeks after administration of a single adriamycin dose. The photomicrograph shows focally destroyed renal tubules, a fragment of renal glomerulus and irregular tubules. Naked nuclei are visible in the lemen. Epithelial cells with brightened cytoplasm and dark distorted pyknotic nuclei are seen. Congestion is visible between tubules. The semi-thin specimen. Methylene blue+Azure II staining. Magnification x 400.



The methods detecting single apoptotic cells as well as their populations [5]

The most common and simple way is to visualize the morphological changes using histological staining. The cell elements visualized by stains, particularly cell nuclei, show different morphology in apoptosis and in necrosis [6]. The cell nucleus is stained with basic (cation) stains such as cresyl violet, haematoxylin, [7], acridine orange, basic fuchsin. The acidophilic cytoplasm is stained with acid (anion) stains: picric acid, acid fuchsin, methyl blue, light green (Fig.2). Fluorescent stains are also commonly used (propidium iodide [8], ethidine bromide, fluorescein).

Semi-thin slides (0.5-0.7μm) stained with methylene blue with Azure II give the possibility to observe single line of cells in the light microscope (Fig. 3).

In the group of methods based on assessment of changes in the apoptotic cell membrane we can distinguish:

- The method making use of the fact that the damaged cell membrane is completely permeable for cation stains. The incubation of cells in the solution of such stains (ethidine bromide, trypan blue, propidium iodide) enables labelling of apoptotic cells.
- The method making use of the fact that phosphatidylserine (PS) contained in the internal cell membrane passes to the external layer in apoptotic cells [9]. The apoptotic cells are labelled with anexin V conjugated with the marker, which binds PS [10].

The other groups of methods are based on Caspase 3 activity examination:

- Substrates of caspase 3 are added to the specimen and its activity is measured by determining the changes in the amount of products of the enzymatic reaction catalyzed by caspase 3.
- WESTERN BLOCK technique; a) electrophoretic separation of the mixture of proteins of tissue homogenate b) transfer of separated components to the carrier, e.g. nitrocellulose - a stable specimen is obtained c) reaction with the anti-caspase 3 antibodies [11].

The apoptois of cells we can detect evaluating the release of cytochrome C from the mitochondria using the monoclonal antibody against cytochrome C. The detection of cytochrome C is preceded by lysis of the cell and separation of mitochondrial and cytoplasmic fractions.

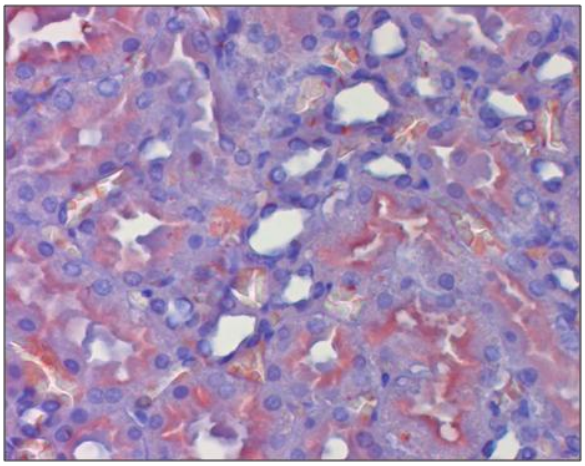
The big group of methods is based on detection of DNA fragmentation [12]. Among other authors describe a way:

- by using electrophoresis on agarose gel
  a) The method based on the difference in DNA molecular weight. The high molecular weight (HMW) DNA fraction occurs in the nuclei of normal cells while low molecular weight (LMW) DNA fraction consists of fragmented DNA in the nuclei of apoptotic cells. The individual fractions are separated by centrifugation [13].
  b) Differentiation of the apoptotic cells from necrotic cells on the basis of the so-called "ladder" composed of histone octamers formed during the electrophoretic separation of DNA from the apoptotic cells. DNA from necrotic cell nuclei is degraded according to a different, unorganized pattern. A „continuous smear" develops.
- *In situ end labelling* (ISEL) [14].
  The method consists in the labelling of DNA breaks by enzymatic attachment of biotin- or digoxygenin (DIG)-conjugated nucleotides to the broken DNA strain.
- In situ nick translation (ISNT) [15].
  The method uses DNA polymerase I showing significant specificity to the substrate, which are uni- and double-strain DNA containing breaks in one strain. There is no specificity to double-strain DNA of proper structure as the role of DNA polymerase I is to mediate in the repair and replication of DNA.
- terminal deoxynucleotydal transferase mediated d-UTP nick end-labelling (TUNEL).
  The method consists in the labelling of DNA nick ends by terminal deoxynucleotidyl transferase (TdT) which incorporates the labelled nucleotide (most often dUTP) in the places of DNA strain breaks [11,14].
- DNA ELISA. The apoptotic cells release nucleosomes to the cytoplasm during DNA degradation. The ELISA test detects single nucleosomes in the cytoplasm [16].

Quite common method is flow cytometry (flow cytophotometry) [17]. This method measures specific physicochemical features of the cells previously labelled with fluorochroms, when they pass the measurement point. The amount of excited fluorescent light is determined, which allows to assess the content of the labelled cells, their structure, shape, size as well as to measure the amount of DNA. The apoptotic cells are recognized thanks to their characteristic features or as the cells with lower DNA content.

Immunohistochemistry, also called immunocytochemistry is the method localizing specific antigens in tissues and cells based on the antigen-antibody reaction. The antigen-antibody binding results in the colour reaction observed under light microscope (Fig. 4).

Fig. 4. The kidney section of the rat treated with ADR and L-ARG. Intensive caspase 3 red reaction. AEC+H staining. Magnification x 400.



Highly specific monoclonal antibodies are produced [18], which provides sufficient sensitivity of the method.

The three-step method is most commonly used:

1. the removal of endogenous peroxidases from the section (peroxidases are oxidized by hydrogen peroxide) and antigen-antibody reaction (antigen reacts with the primary antibody),

2. the reaction of primary antibody with biotinylated secondary antibody,
3. the reaction of biotin with avidin (ABC-avidin-biotin complex) or streptavidin (BSA-biotin-streptavidin) conjugated with horse-radish peroxidase.

The addition of the dye (AEC or DAB) which is oxidized by horse-radish peroxidase at the site of antigen-antibody reaction gives red (AEC 3-amino-9-ethylcarbazole) or brown (DAB) stain at the site of occurrence of the antigen looked for.

References

1. Bielak-Żmijewska A. Mechanizmy oporności komórek nowotworowych na apoptozę. Kosmos. Problemy nauk biologicznych, 2003; 52: 157-171.
2. Pryjma J. Monocyty krwi obwodowej – apoptoza po fagocytozie bakterii oraz amiany funkcjonalne w następstwie kontaktu z komorkami apoptotycznymi www. fnp.org.pl/programy aktualne/jachranka/profPryjma.pdf. 2004; 1-19.
3. Chiba T., Hwang B.H., Williams T.H. A method for studying glyoxylic acid induced fluorescence and ultrastructure of monoamine neurons. Histochemistry, 1976; 49: 95-106.
4. Lee R.F., Steinert S.A. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutat Res, 2003; 544: 43-64.
5. Sulejczak D. Apoptosis and methods of identification of thi phenomenon. Post Biol Kom, 2000; 27: 527-568.
6. Solomon S., Mpoke S.S., Wolfe J. Differential Staining of Apoptotic Nuclei in Living Cells: Application to Macronuclear Elimination in Tetrahymena. J. Histochem. Cytochem, 1997; 45: 675-684.
7. Smith S.C., Leung T.N., To K.F., Baker P.N. Apoptosis is a rare event in first-trimester placental tissue. Am. J. Obstet. Gynecol, 2000; 183: 697-699.
8. Verda L. Wpływ cytostatyków na apoptozę limfocytów w przewlekłej białaczce limfatycznej B-komórkowej w badaniach in vitro. Praca Doktorska synaba Coll Med. UJ, 2001.
9. Fadok V., Bratton D.M., Rose A., Pearson R., Alan B., Ezekewitz P.M., Henson P.M. A receptor for fosfatidyloserine – specific clearance of apoptotic cells. Nature, 2000; 405: 85-90.
10. Kirszberg C., Rumjanek V.M., Capella M.A. Methylene blue is more toxic to erythroleukemic cells than to normal peripheral blood mononuclear cells: a possible use in chemotherapy. Cancer Chemother Pharmacol, 2005; 56: 659-665.
11. Peng Y., Gallagher S.F., Haines K., Baksh K., Murr M.M. Nuclear Factor-kappa B Mediates Kupffer Cell Apoptosis Through Transcriptional Activation of Fas/FasL. J Surg Res, 2006; 130: 58-65.
12. Arends M.J., Morris R.G., Wyllie A.H. Apoptosis: the role of the endonuclease. Amer J Pathol, 1990; 136: 593-608.
13. Yuste V.J., Sanchez-Lopez I., Sole C., Moubarak R.S., Bayascas J.R., Dolcet X., Encinas M., Susin S.A., Cornella J.X. The contribution of apoptosis-inducing factor, caspase-activated DNase, and inhibitor of caspase-activated DNase to the nuclear phenotype and DNA. J Biol Chem, 2005; 280: 35670-35683.
14. Lesauskaite V., Epistolato M.C., Ivanoviene L., Tanganelli P. Apoptosis of cardiomyocytes in explanted and transplanted hearts. Comparison of results from in situ TUNEL, ISEL, and ISOL reactions. Am J Clin Pathol, 2004; 121: 108-116.
15. Enciso M., Lopez-Fernandez C., Fernandez J.L., Garcia P., Gosalbez A., Gosalvez J. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology, 2006; 65: 308-316.
16. Salgame P., Varadhachary A.S., Primiano L.L., Fincke J.E., Muller S., Monestier M. An ELISA for detection of apoptosis. Nucleic Acids Res, 1997; 25: 680-681.
17. Jimenez R., Carracedo J., Santamaria R., Soriano S., Madueno J.A., Ramirez R., Rodriguez M., Martin-Malo A., Aljama P.: Replicative senescence in patients with chronic kidney failure. Kidney Int, 2005; 68: S11-S15.
18. Kohler G., Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. J Immunol, 2005; 174: 2453-2455.

Correspondence address

Agnieszka Pedrycz
Department of Histology and Embryology Medical University in Lublin, ul. Radziwiłłowska 11, 20-080 Lublin
e-mail address: apw4@wp.pl;
tel. 81 528 84 04