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review

Detection of apoptotic cells in tumour paraffin sections

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Apoptosis is a distinct form of cell death characterised by specific morphological features and regulated by complex molecular mechanisms. Its deregulation is fundamental for tumour growth and progression and, moreover, anticancer therapies suppress tumour growth mainly by induction of apoptosis. Since the extent of apoptosis in a tumour may have prognostic as well as therapeutic implications, much effort has been invested in developing specific methods that can be routinely used to detect apoptotic cells in archival formalin-fixed paraffin-embedded tissue.

Complex molecular pathways are involved in the regulation of apoptosis. Pro-apoptotic signals trigger activation of caspases that specifically cleave target proteins. Cleavage of proteins (caspase substrates) is responsible for morphological changes of apoptotic cells and DNA fragmentation. In the last decade, detection of apoptotic cells in formalin-fixed tumour tissue sections has been based mainly on morphology and characteristic DNA fragmentation. Recently, specific antibodies to activated caspases and cleaved target proteins (including cytokeratin 18, actin and PARP) have been produced that enable accurate detection of apoptosis in paraffin sections.

Key words: neoplasms; apoptosis; caspases; DNA fragmentation

Introduction

Apoptosis, or programmed cell death, is a complex, tightly regulated and conserved process, whereby individual cells die without injuring neighbouring cells or provoking any inflammatory reaction. It is essential for the maintenance of homeostasis in multicellular organisms and is a critical component in the cellular response to injury.[1] Apoptosis is implicated in the patogenesis of various conditions, such as cancer, autoimmune disorders and neurodegenerative diseases. An imbalance between cell proliferation and apoptosis is fundamental for tumour growth and progression. Because of its wide ranging implications and possible therapeutic interventions, apoptosis is one of the most investigated areas in biological science.[2]

Since various proteins implicated in apop-

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tosis regulation have been reported to be mutated in cancer, and it has become evident that different anticancer therapeutic modalities suppress tumour growth mainly by induction of apoptosis, accurate quantification of the extent of apoptosis in tissue specimens may have therapeutic as well as prognostic implications.[3,4]

Many different biochemical, cytochemical and *in situ* methods are available for apoptosis detection,[5-8] but only a limited number of them are applicable on paraffin sections. Because of the preserved cell morphology in archival formalin-fixed paraffin-embedded tissue, there has been considerable interest in developing methods that could specifically demonstrate apoptotic cells in tissue sections. In the last decade, the morphological method and the TUNEL method (terminal transferase mediated d-UTP biotin nick end labelling) have been almost exclusively used for apoptosis detection on paraffin sections. Since great progress in understanding the mechanisms of apoptosis has been made, several new methods for apoptosis detection on paraffin sections have been introduced recently. This article deals with apoptosis detection techniques applicable to archival paraffin sections with an emphasis on tumour tissue.

Apoptotic pathway

Initially, apoptosis was recognised on morphological grounds as a well defined type of cell death.[9] During apoptosis, the cell shrinks, it detaches from the surrounding cells, the nucleus becomes condensed and fragmented, and finally, apoptotic bodies are formed. In the last two decades, the molecular basis of apoptosis has been elucidated. It has become apparent that there is a cascade of events from pro-apoptotic signalling to well defined morphological changes.

Caspases (cysteine proteases that cleave their substrates following an aspartate residue) have been recognised as key molecules in the apoptotic cascade.[5,10] At least 14 different caspases have been characterised to date. They are constitutively expressed as inactive pro-enzymes (pro-caspases) in virtually all animal cells. Pro-caspases (32 - 56 kDa) contain a large subunit (17 - 21 kDa), a small subunit (10 - 13 kDa) and an N-terminal prodomain. Pro-caspase activation requires proteolytic cleavage of the pro-caspase chain at two caspase specific cleavage sites, yielding a large and a small subunit. Active caspase is a heterotetramer composed of two large and two small subunits.

In mammalian cells, apoptosis can be initiated by either intracellular or extracellular pathways that induce initiator caspase activation (caspases 8 and 9). Activated caspase 8 and/or 9, in turn, induce cascade activation of effector caspases (caspases 3, 6 and 7), which cleave different target proteins. Demolition of the target proteins induces DNA fragmentation and is responsible for morphological changes of the apoptotic cells (Figure 1).

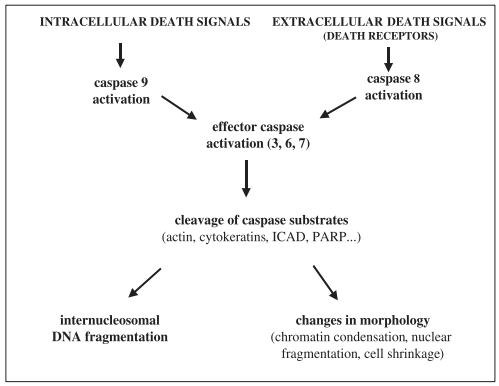

Figure 1. Apoptotic pathway. Different pro-apoptotic signals converge to induce activation of the caspase cascade. Activation of initiator caspases (8 and 9) is followed by activation of effector caspases which, in turn, specifically cleave different target proteins (caspase substrates). Cleavage of the target proteins induces DNA fragmentation and provokes morphological changes of the apoptotic cells.

Detection of apoptotic cells based on morphology

Apoptotic cells can readily be identified by means of routine histological staining methods, such as haematoxylin and eosin. Detection of apoptotic cells is based on characteristic morphological features.[11] The cytoplasm of apoptotic cells is condensed and eosinophilic. Chromatin is condensed, marginated at the nuclear membrane, and nuclear fragments are later seen (Figure 2a). Finally, the apoptotic cells desintegrate into membrane-bound apoptotic bodies, which are phagocytosed by neighbouring cells and macrophages.[12] During apoptosis, the cell membrane retains its integrity and lysosomal enzymes are not released to the surrounding tissue, so no inflammatory reaction is elicited. All morphological features are best viewed by electron microscope, but this is impractical for screening large tissue areas.[13]

Visible changes in cell morphology are the final event of the apoptotic process and are estimated to take from two to three hours, compared to 12 to 24 hours that are needed for the entire apoptotic process to be completed.[12,14] The basis for changes in the apoptotic cell morphology is caspase mediated cleavage of the target proteins (Figure 1).

The asynchronicity of apoptosis, completion of the morphological phase in a few hours and immediate clearing from the tissue usually results in a very low number of apoptotic cells in tissues.[11] Because morphological changes may be inconspicuous, the lowest numbers of apoptotic cells are usually detected by morphological criteria only.[12]

An apoptotic index is used as a measure of the extent of apoptosis, which is defined in tumour tissues as the percentage of apoptotic cells of all tumour cells. Because apoptotic cells frequently appear in clusters, enough fields need to be included in the analysis. To guarantee representativeness, at least 20 fields of 1000x magnification should be examined.[15] The identification of apoptotic cells, and thus inter-observer variation, greatly depends on the magnification, so a higher magnification should be used. Inflammatory cells that are frequently found among tumour cells may substantially influence the apoptotic index. They may be difficult to differentiate from apoptotic cells (inflammatory cells are also frequently apoptotic) as well as from the population of tumour cells analysed.[12]

Generally, assessing apoptosis based solely on its morphology is reasonably reliable and inexpensive, although tedious and fairly inter-observer dependent. It might be accurate for some tissues,[15-17] but for more accurate assessment, morphology should be used in combination with more specific methods, which detect apoptotic cells earlier in the apoptotic process. However, morphology based on routine staining methods remains, even today, a major tool of apoptosis detection and is critical for validation of new techniques.

Detection of DNA fragmentation

In situ detection of DNA fragmentation has been the most widely used method for detection of apoptosis.[12] The orderly internucleosomal fragmentation of DNA into 180 to 200 base-pair fragments is the biochemical hallmark of apoptosis.[16] It is induced by caspase mediated cleavage of ICAD (*inhibitor of CAD*) leading to activation of CAD (caspase-activated deoxyribonuclease), which in turn, cleaves DNA.[18]

DNA fragments can be detected by enzymatic labelling of the 3'-hydoxyl ends with modified nucleotides. The most sensitive and specific *in situ* method for detection of DNA fragmentation is the TUNEL method (terminal deoxynucleotidyl transferase-mediated-dUTP nick end labelling) (Figure 2b). It was introduced for use in tissue sections in 1992 by Gavrieli et al.[19] As its name implies, the TUNEL method is based on the addition of la-

belled nucleotides to free 3'-hydoxyl ends of single or double-strand DNA breaks catalysed by a terminal deoxynucleotidyl transferase (TdT) enzyme. Following *in situ* enzyme reaction, the incorporated labelled nucleotides can be detected by immunohistochemistry. Nuclear counterstains are used to facilitate recognition of apoptotic morphology.[11]

There are many commercially available apoptosis detection kits that rely on detection of DNA fragments.[16]

TUNEL labelling greatly depends on tissue fixation, tissue pretreatment and the concentration of TdT. Overlong formalin fixation and insufficient pretreatment may give rise to false negative results. On the other hand, excessive pretreatment, necrosis, autolysis or extensive DNA repair may lead to false positive results.[12] In some tissues, proteinase pretreatment may result in release of endogeneous endonucleases, which may cause false positive results with TUNEL staining.[20] Generally, TUNEL gives higher apoptotic indices than the morphological method, at least partly because DNA fragmentation slightly precedes light microscopic morphological changes.[14] TUNEL has been the most widely used method for apoptosis detection on tumour material. However, there are some drawbacks that warrant caution. TUNEL is an *in situ* enzymatic method followed by an immunohistochemical reaction and, due to its complexity, the results depend greatly on tissue preparation, pretreatment and reaction conditions.[12] Optimisation of TUNEL staining therefore requires standardisation of the above mentioned technical factors. Moreover, DNA fragmentation is a late event in apoptosis, it is not an obligatory feature of apoptosis, and may be absent.[16]

Immunohistochemical detection of cleaved caspase 3

As a result of increased knowledge about the molecular mechanisms of apoptosis, detection of apoptotic cells based on morphology and detection of DNA fragmentation is now advancing to more specific methods. Proteins selectively activated, or protein fragments generated during the process of apoptosis, have been characterised, and specific antibodies against them allow reliable detection of apoptotic cells, distinguishing them from necrotic cells.[11]

Caspase 3 is activated during most apoptotic processes and is believed to be the main effector caspase. Its activation is directly or indirectly responsible for cleavage of the target proteins, which leads to characteristic DNA fragmentation and morphological changes of apoptotic cells.[21] The appearance of the active form of caspase 3 in the cell undergoing apoptosis is an early event during apoptosis and precedes the development of classical morphological features. At the time of caspase 3 activation, the cell is fully committed to death and apoptosis is said to run beyond the 'point of no return'.[22] Detection of cleaved caspase 3 enables detection of apoptosis even before the morphological changes of apoptosis appear.

Unprocessed caspase 3 is a 32 kDa protein which is cleaved by upstream caspases into 17 kDa and 12 kDa active fragments.[5] This cleavage creates or unmasks new epitopes. Specific antibodies are available that recognise specifically the large subunit of processed caspase 3, but do not react with unprocessed caspase 3 and are therefore specific for the active caspase 3 (Figure 2c). These antibodies have been shown to work on paraffin sections.[11,23,24] Processed caspase 3 is a highly specific marker for apoptosis, since no activation of the caspase cascade has been found in necrotic cells.[11] The detection of active caspase 3 in cells can therefore be used as a discriminating criterion to distinguish apoptosis from necrosis.

Different studies have confirmed the usefulness of antibodies to cleaved caspase 3 in detecting apoptotic cells in non-tumour as well

in tumour tissues.[23-25] In breast tissue, a strong correlation between the apoptotic index assessed by morphology in haematoxilin and eosin stained sections and the apoptotic index assessed using antibodies to cleaved caspase 3 has been reported. The immunohistochemical reaction makes recognition of apoptotic cells easier and reduces subjectivity in interpretation, thus reducing inter-observer variability.[24]

One study investigating apoptosis detection in normal tissues, showed prominent immunostaining to cleaved caspase 3 in germinal centres and in neutrophilic granulocytes.[23] A prominent granulocyte infiltration can be found in tumour tissues. In our experience, many (but not all) granulocytes are positive to cleaved caspase 3. It is not clear whether this represents specific detection of apoptosis of granulocytes. However, positive granulocytes should not be confused with tumour apoptotic cells.

In some tumours, no cleaved caspase 3 could be detected. This might reflect a low level of apoptotic activity, but the expression of uncleaved caspase 3 may nevertheless be downregulated in tumours.[26]

Immunohistochemical detection of caspase-cleaved target proteins

It has become evident that caspase mediated cleaveage of target proteins at specific consensus sequences represents a unique feature of apoptosis. At least 100 different caspase substrates have been identified. Among them are structural proteins, such as actin and cytokeratins, cell signalling molecules, regulators of cell cycle and DNA repair, such as PARP (polyADP-ribose polymerase), regulators of cell-cell interactions, inhibitors of endonucleases, such as ICAD, and others.[5,27] Their cleavage can result in their inactivation or activation, leading to morphological changes of apoptotic cells and DNA fragmentation.

Cleavage of target proteins may generate or unmask new epitopes. The generation of antibodies to such epitopes, which recognise cleaved but not uncleaved target proteins, has been reported for several target proteins.

Cytokeratins are intermediary filaments of epithelial cells. Different cytokeratins are found in different epithelial cells as well as in epithelial tumours, so they are considered differentiation markers for epithelia and epithelial tumours.[28] Cytokeratin 18 is expressed in simple nonstratified, ductular and pseudostratified epithelia (hepatocytes, renal tubular cells, ductular epithelia, mesothelium, respiratory epithelium).[16,29] Caspase mediated cleavage of cytokeratin 18 leads to exposure of an epitope that can be recognised by the binding of specific antibodies. The M30 antibody recognises cleaved but not uncleaved cytokeratin 18.[5,29] This antibody works on paraffin sections and has been shown to detect apoptotic cells in neoplastic simple epithelia as well in lung and colonic carcinoma. Detection of cleaved cytokeratin 18 precedes TUNEL positivity, indicating that cleavage of cytokeratin represents an early event, but in the late phase of apoptosis, the M30 epitope is lost. Its use is, however, limited to detecting apoptotic cells in epithelial tissues or tumours expressing cytokeratin 18.

Actin filaments are present in all human cells. During apoptosis, caspase-mediated cleavage of actin generates an actin fragment, which is specifically recognised by a polyclonal antibody, called fractin.[30] Positive fractin immunostaining of apoptotic cells has been shown in several neoplastic and non-neoplastic tissues. Fractin preferentially stains apoptotic bodies, indicating that caspase cleavage of actin filaments occurs late in the apoptosis.

PARP (poly ADP-ribose polymerase) is a 116 kDa nuclear protein that is implicated in DNA repair. During apoptosis, it is one of the earliest proteins cleaved (and thus inactivated) by caspases.[31] PARP cleavage generates an 89 kDa C-terminal fragment and a 24 kDa

N-terminal peptide. The 89 kDa fragment can be detected by the use of specific antibodies (Figure 2d).[32] PARP degradation may precede DNA cleavage, as evidenced by the presence of the 89 kDa fragment in TUNEL negative cells. Therefore, in addition to late apoptosis (TUNEL positive), antibodies against cleaved PARP can detect early apoptosis before DNA cleavage.[33]

Conclusions

Three decades ago, based on the morphology, apoptosis was recognised as a special type of cell death. Later, fragmentation of nuclear DNA was recognised as a biochemical hallmark of apoptosis, and methods for detection of DNA fragments were developed. Recently, the apoptotic pathway has been elucidated and caspases have been recognised as key regulatory and executioner molecules of apoptosis. Their activation, followed by the cleavage of target proteins, precedes and is responsible for DNA fragmentation and morphological changes of apoptotic cells. Based on this knowledge, new more specific and sensitive *in situ* immunohistochemical methods for the detection of apoptotic cells have been developed that enable accurate and rou-

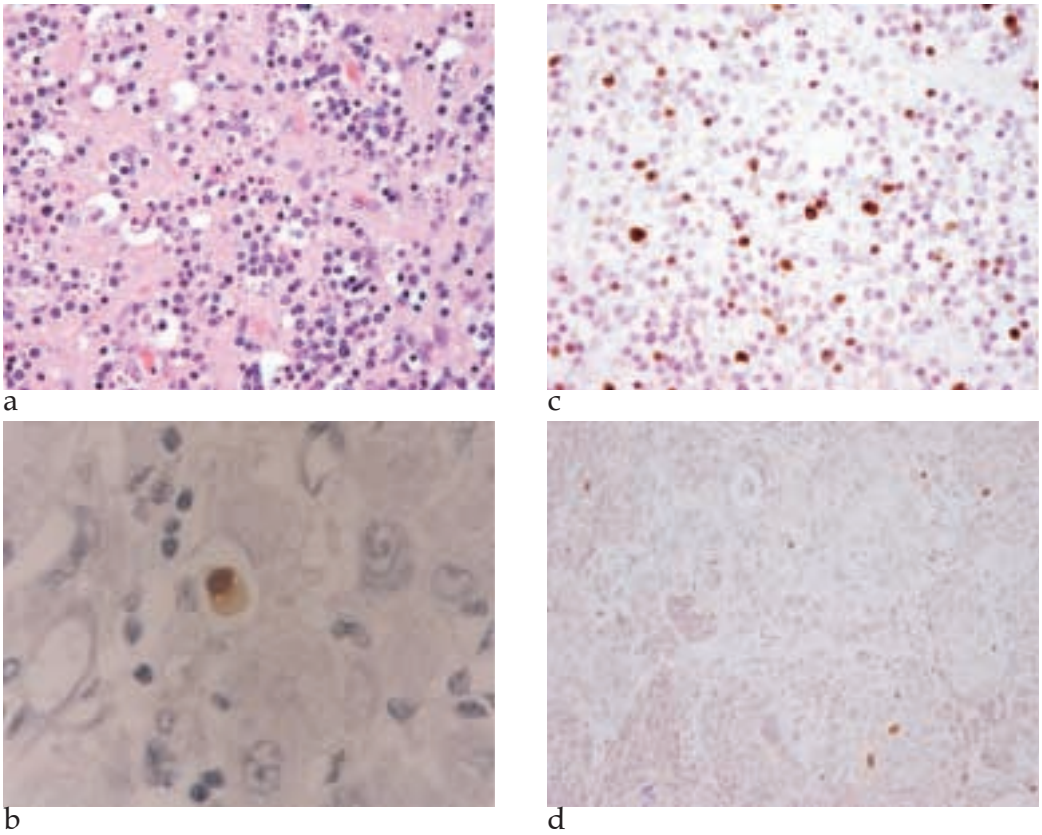

Figure 2. Apoptotic cells in tumour tissues. (a) Numerous apoptotic cells in multiple myeloma, as revealed by condensed eosinophilic cytoplasm and condensed and fragmented nuclei in haematoxylin and eosin sections. (b) An apoptotic cell in hepatocellular carcinoma labelled by the TUNEL method. (c) Apoptotic cells immunostained against active caspase 3, the same tumour tissue as in (a). (d) Apoptotic cells in squamous cell carcinoma immunostained against cleaved PARP. Magnification 400x a,c,d and 1000x b.

tine detection of apoptosis on formalin-fixed and paraffin-embedded tissue.

References

1. Raff M. Cell suicide for beginners. *Nature* 1998; **396**: 119-22.
2. Gastman BR. Apoptosis and its clinical impact. *Head Neck* 2001; **23**: 409-25.
3. Ryungsa K, Kazuaki T, Yoko U, Manabu E, Hideki I, Tetsuya T. Current status of the molecular mechanisms of anticancer drug-induced apoptosis. *Cancer Chemother Pharmacol* 2002; **50**: 343-52.
4. Dukers DF, Meijer CJ, ten Berge RL, Vos W, Ossenkoppele GJ, Oudejans JJ. High numbers of caspase 3-positive Reed-Sternberg cells in pretreatment biopsy specimens of patients with Hodgkin disease predict favourable clinical outcome. *Blood* 2002; **100(1)**: 36-42.
5. Köhler C, Orrenius S, Zhivotovsky B. Evaluation of caspase activity in apoptotic cells. *J Immunol Methods* 2002; **265**: 97-100.
6. Willingham MC. Cytochemical methods for the detection of apoptosis. *J Histochem Cytochem* 1999; **47(9)**: 1101-9.
7. Castedo M, Ferri K, Roumier T, Metivier D, Zamzami N, Kroemer G. Quantification of mitochondrial alterations associated with apoptosis. *J Immunol Methods* 2002; **265**: 39-47.
8. Van Heerde WL, Robert-Offerman S, Dumont E, Hofstra L, Doevendans PA, Smits JF et al. Markers of apoptosis in cardiovascular tissues: focus on Annexin *V. Cardiavasc Res* 2000; **45**: 449-59.
9. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basis biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**: 239-57.
10. Pižem J, Cör A. Kaspaze. *Med Razgl* 2001; **40**: 283-91.
11. Stadelmann C, Lassmann H. Detection of apoptosis in tissue sections. *Cell Tissue Res* 2000; **301**: 19-31.
12. Soini Y, Paakko P, Lehto V-P. Histopathological evaluation of apoptosis in cancer. *Am J Pathol* 1998; **153**: 1041-53.
13. Cummings MC, Winterford CM, Walker NI. Apoptosis. *Am J Surg Pathol* 1997; **21(1)**: 88-101.
14. Elsässer A, Suzuki K, Lorenz-Meywe S, Bode C, Schaper J. The role of apoptosis in myocardial ishemia: a critical appraisal. *Basic Res Cardiol* 2001; **96**: 219-26.
15. Van de Schepop HAM, de Jong JS, van Diest PJ, Baak JPA. Counting of apoptotic cells: a methodological study in invasive breast cancer. *J Clin Pathol Mol Pathol* 1996: **49**: 214-17.
16. Alison MR. Identifying and quantifying apoptosis: a growth industry in the face of death. *J Pathol* 1999; **188**: 117-8.
17. Pižem J, Ferlan-Marolt V, Luzar B, Cör A. Proliferative and apoptotic activity in hepatocellular carcinoma and surrounding non-neoplastic liver tissue. *Pflügers Arch* 2000; **422(6) [Suppl1]**: 174-6.
18. Zheng TS, Hunot S, Kuida K, Flavell RA. Caspase knockouts: matters of life and death. *Cell Death Differ* 1999; **6**: 1043-53.
19. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J cell Biol* 1992; **119(3)**: 493-501.
20. Stäheliin BJ, Marti U, Solioz M Zimmermann H, Reichen J. False positive staining in the TUNEL assay to detect apoptosis in liver and intestine is caused by endogenous nucleases and inhibited by diethyl pyocarbonate. *J Clin Pathol Mol Pathol* 1998; **51**: 204-8.
21. Porter AG, Jänicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ* 1999; **6**: 99-104.
22. Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ* 1999; **6**: 1067-74.
23. Gown AM, Willingham MC. Improved detection of apoptotic cells in archival paraffin sections: immunohistochemistry using antibodies to cleaved caspase 3. *J Histochem Cytochem* 2002; **50(4)**: 449-54.
24. Hadjiloucas I, Gilmore AP, Bundred NJ, Streuli CH. Assessment of apoptosis in human breast tissue using an antibody against the active form of caspase 3: relation to tumour histopathological characteristics. *Br J Cancer* 2001; **85(10)**: 1522-6.
25. Urase K, Fujita E, Miho Y, Kouroku Y, Mukasa T, Yagi Y et al. Detection of activated caspase-3 (CPP32) in the vertebrate nervous system during development by cleavage site-directed antiserum. *Develop Brain Res* 1998; **111**: 77-87.
26. Cör A, Pižem J, Gale N. The expression of Bcl-2 and pro-caspase 3 in head and neck squamous cell carcinoma. *Zdrav Vest* 2002; **71(Suppl III)**: 39-44.

27. Nicholson DW. Caspase structure, photolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 1999; **6**: 1028-42.

28. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathol* 2002; **40**: 403-39.

29. Leers MPG, Kölgen W, Björklund V, Bergman T, Tribbick G, Persson B et al. Immunocytochemical detection and mapping of cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* 1999: **187**: 567-72.

30. Suurmeijer AJH, van der Wijk J, van Veldhuisen DJ, Yang F, Cole GM. Fractin immunostainig for the detection of apoptotic cells and apoptotic bodies in formalin-fixed and paraffin-embedded tissue. *Lab Invest* 1999; **79(5)**: 619-20.

31. Duriez PJ, Shah GM. Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem Cell Biol* 1997; **75(4)**: 337-49.

32. Dukers DF, Oudejans JJ, Vos W, ten Berge RL, Meijer CJ. Apoptosis in B-cell lymphomas and reactive lymphoid tissues always involves activation of caspase 3 as determined by a new in situ detection method. *J Pathol* 2002; **196(3)**: 307-15.

33. Soldani C, Bottone MG, Pellicciari C, Scovassi AI. Two-color fluorescence detection of Poly (ADP-Rybose) Polymerase (PARP-1) cleavage and DNA strand breaks in etoposide-induced apoptotic cells. *Eur J Histochem* 2001; **45(4)**: 389-92.