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Detection of apoptosis in vivo using antibodies against caspase-induced neo-epitopes

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Abstract

Cell death induction by apoptosis is an important process in the maintenance of tissue homeostasis as well as tissue destruction during various pathological processes. Consequently, detection of apoptotic cells in situ represents an important technique to assess the extent and impact of cell death in the respective tissue. While scoring of apoptosis by histological assessment of apoptotic cells is still a widely used method, it is likely biased by sensitivity problems and observed-based variations. The availability of caspase-mediated neo-epitope-specific antibodies offers new tools for the detection of apoptosis in situ. Here, we discuss the use of immunohistochemical detection of cleaved caspase 3 and lamin A for the assessment of apoptotic cells in paraffin-embedded liver tissue. Furthermore, we evaluate the effect of tissue pretreatment and antigen retrieval on the sensitivity of apoptosis detection, background staining and maintenance of tissue morphology.

Keywords: Apoptosis; Liver; Caspase; Lamin A; Fas/CD95; Immunohistochemistry; Paraffin sections; Histology; Antigen retrieval

1. Introduction

Cell death has been recognized as essential mechanism to counter-balance cell division and proliferation in order to maintain tissue homeostasis. Aberrant excessive cell death has been implicated in various forms of disease-associated tissue damage whereas reduced cell death is a typical feature of cancerous tissues [1,2]. Apoptosis represents a defined form of a tightly controlled, programmed cell death, distinct from the accidental death occurring during necrosis. It was been originally described as a specific and morphologically distinct form of cell death in various tissues [3,4]. Consequently, the morphological assessment of apoptotic cells by light or electron microscopy has been for a long time one of the gold standards of in situ apoptosis detection and is still frequently used to describe apoptotic cell death in tissues. However, scoring of apoptosis on tissue sections using routine histological stainings is tedious and observer-dependent [3,5]. In addition, characterization of the different biochemical processes, leading to the distinct morphological changes of apoptotic cells, i.e. cellular and nuclear condensation and fragmentation, has pointed out that these morphological changes occur relatively late after cell death induction. Furthermore, apoptotic cells display relatively early on different eat-me signals, resulting in rapid phagocytosis and the removal of apoptotic cells [6]. Consequently, a scoring of apoptotic cell death in tissue sections solely based on morphological changes might largely underestimate the real frequency of dying cells. A classical example for the inefficient detection of apoptosis by morphological techniques, respectively efficient removal of apoptotic cells, is the thymus where approximately 95% of all thymocytes die by apoptosis, yet only a minor fraction is observed as displaying apoptotic morphologies [7–9].

This clearly demonstrates the need of more sensitive methods to detect apoptotic cells in tissue sections. Such

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enhanced morphology-based detection systems are not only important for in vivo apoptosis studies in basic research, but represent also important diagnostic tools for pathologists and disease diagnosis. Not surprisingly, reduced or increased apoptotic cell death in tissue specimens has been associated with various diseases and correlations with severity of disease and/or patients survival have been demonstrated [10–12].

Early on in the characterization of apoptosis DNA fragmentation has been recognized as one of the hallmarks of apoptosis [9]. Since its introduction [13,14] the TUNEL (terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling) assay has been extensively used for the detection of apoptosis-associated DNA strand breaks in cultured cells and tissue sections. While the TUNEL technique has certain advantages, e.g. that it can be combined with immunohistochemistry or other antibody-based techniques [15], it has also various disadvantages. A major problem is the fact that DNA fragmentation is not restricted to apoptosis but also occurs in necrotic cells, only the frequency of DNA strand breaks is increased in apoptotic cells. Similarly, overfixation of tissue samples in formalin may cause DNA breaks, which are further exposed after pretreatment of tissue sections with proteinase K to allow the terminal deoxynucleotide transferase get access to the nuclei. Finally, the extent and duration of proteinase K digestion largely defines the sensitivity and the background of this technique. Thus, TUNEL is considered a technically demanding apoptosis detection method for experienced users, requiring a large panel of positive and negative controls [5,16].

The aspartate-specific cysteine proteases, caspases, are key effector molecules of apoptotic cell death [17–19]. Caspases are expressed in most cells and activated upon most apoptotic stimuli. Importantly, the typical morphologic changes occurring in apoptotic cells largely depend on caspase activity. Current estimates propose up to 400 different caspase substrates, which become proteolytically cleaved during apoptosis, and cleavage of some substrates has been correlated with specific morphological changes occurring in apoptotic cells [20,21]. Caspase activation and substrate cleavage can be easily monitored in vitro by detecting their processing using Western blots and specific antibodies [22]. These techniques are however not applicable to in situ apoptosis detection on tissue sections. The discovery that caspases and caspase substrates are cleaved at very specific sites leading to neo-epitopes with defined amino acid sequences has led to the generation of antibodies reacting only with cleaved (activated) caspases and caspase substrate [23,24]. As the generation of these neo-epitopes is dependent on apoptosis induction and caspase activation, such antibodies represent useful and valuable tools for detecting apoptosis-associated processes in cell culture and on tissues sections [5].

Currently, numerous antibodies against various cleaved (active) caspases or cleaved caspase substrates are commercially available and have been used for apoptosis detection in situ [23–25]. However, not all caspases or caspase substrates may be equally well expressed and/or cleaved in different cell types or after apoptosis induction by different triggers. Caspase 3 is a critical effector caspase, expressed in most cells and activated in response to most apoptosis triggers [22]. Similarly, lamin A is a ubiquitously expressed nuclear protein, which is cleaved in a caspase-dependent manner during apoptosis [26,27]. In this study we describe the use of anti-cleaved caspase 3 and anti-cleaved lamin A for the detection of apoptosis in formalin-fixed mouse liver tissue using immunohistochemistry. Specifically, we address the role of tissue pretreatments and antigen retrieval on the sensitivity of apoptosis detection and maintenance of tissue morphology. We exclusively focused on paraffin-embedded tissue as the histology is preserved much better than in cryosections and paraffin-embedded tissue is often more readily available, e.g. in tissue banks and diagnostic archives.

2. Materials and methods

2.1. Induction of apoptosis in mouse liver tissue

Apoptosis induction in murine liver tissue was performed as described previously by Nagata and colleagues [28], and modified by Corazza et al. [29]. The injection of the agonistic anti-Fas (CD95) antibody (clone Jo-2) leads to rapid stimulation of the Fas receptor and induction of massive hepatocyte apoptosis. Female C57Bl/6 mice (7–10 weeks) were weighted to calculate the antibody/body weight ratio, and immobilized in a mouse strainer. Anti-Fas antibody (0.25 μg/g body weight, clone Jo-2, e-bioscience, San Diego, CA) or PBS control was injected i.v. into the tail vein using a 1 ml tuberculin syringe and a 27G needle. Control animals received i.v. injection of PBS only. After 4 h mice were euthanized with a CO_2 overdose, liver samples were immediately resected and fixed overnight in 4% paraformaldehyde (Sigma) in PBS. Fixed liver tissue was then dehydrated in an increasing ethanol row and embedded in paraffin using a Tissue Tek II Tissue Embedding Center (Haska, Bern, Switzerland). Five um sections were then cut on a Jung SM 200R microtome (Leica, Nussloch, Germany) and mounted on microscope glass slides (Superfrost Plus, Thermo Fisher Scientific). Sections were dried for 2 h at 57 °C, and deparafinized and rehydrated for 2×10 min in xylole, 5 min in 100% ethanol, and then for 20 s each in 95%, 95%, 70%, 70%, 35% ethanol, H_2O and 25 mM TBS pH 7.5.

2.2. Pretreatments (antigen retrieval)

Fixation of the tissue leads to a variable degree to the denaturation and crosslinking of the antigen of interest with other proteins. This leads to antigen masking and the inability of the antibody to recognize the antigen. As a consequence various tissue pretreatment methods have been developed that lead to the retrieval of the antigen.