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Dual Detection of Nucleolytic and Proteolytic Markers of Lysosomal Cell Death: DNase II-Type Breaks and Cathepsin D

Candace L. Minchew and Vladimir V. Didenko

Abstract

Lysosomes contain hydrolytic enzymes that can degrade proteins and DNA. Leakage of these reactive compounds through a compromised lysosomal membrane causes lysosomal cell death, which can have apoptotic, necrotic, or mixed morphology. Lysosomal cathepsin proteases, such as cathepsin D, and the lysosomal endonuclease, DNase II, have both been implicated in lysosome-related cell death. Here, we present a fluorescence dual-labeling technique for simultaneous visualization of these two markers of lysosomal activity linked to cell death. The approach labels the intracellular distribution of cathepsin D and the sites with DNase II-type breaks in fixed tissue sections. It determines the lysosomal or extra-lysosomal localization of the markers and can be useful in studying pathways and signals of lysosomal cell death.

Keywords

Lysosomal cell death; DNase II-type breaks; Cathepsin D; Fluorescence dual-labeling technique; Vaccinia topoisomerase-based labeling; DNase II; DNA breaks; Phagolysosomes; Lysosomes; Clearance of apoptotic cells

1 Introduction

Lysosomes are membrane-bound catabolic organelles, ranging in size from ~100 to 1200 nm and carrying multiple hydrolytic enzymes [1]. Lysosomes contain about 50 different acid hydrolases [2], including the highly active acid deoxyribonuclease II (DNase II) and cathepsin D protease. Such numerous and concentrated hydrolytic enzymes pose a potential danger to a cell.

Cells employ two main mechanisms for protection from the harmful activity of lysosomal hydrolases. First, the cellular cytosol has a pH of ~7.2 which suppresses the activity of these enzymes, as they are optimally active at an acidic pH [3]. Second, the lysosomal membrane serves as a protective barrier, preventing lysosomal hydrolases from entering the cytosol.

However, both of these protective mechanisms are not absolute. The lysosomal membrane can be damaged or permeabilized by environmental changes or chemical agents. In such cases, lyso-somal contents are released into the cytosol, damaging vital cellular components. This initiates the lysosomal cell death pathway [4, 5]. Depending on the extent of the release and the massiveness of the damage, a cell can either die by necrosis or proceed to apoptotic-type disassembly [4].

Among different enzymes released into the cytosol after lysosomal membrane permeabilization, two attracted particular attention: cathepsin D protease and DNase II nuclease.

1.1 Cathepsin D Lysosomal Protease

Cathepsin D is a ubiquitously expressed lysosomal endopeptidase that can cleave a variety of protein substrates. Studies demonstrated that active forms of cathepsin D can be released from lysosomes into the cytosol after lysosomal membrane permeabilization and in response to various apoptotic signals. Such cytoplasmic cathepsin D induces lysosomal cell death [6]. Cathepsin D retains a portion of its protease activity at a neutral cytoplasmic pH, but can trigger cell death even if it is enzymatically inactive [5–7]. Therefore, determining intracellular distribution of this protease is important for studies of lysosome-triggered cell death.

1.2 DNase II Lysosomal Nuclease

DNase II is the primary lysosomal endonuclease, responsible for the digestion of the engulfed DNA. The single nuclease with DNase II characteristics is present in lysosomes and is absolutely required for engulfment-mediated DNA digestion [8, 9] (*see Note 1*). DNase II efficiently cuts both double-stranded and single-stranded DNA. Although it is optimally active at an acidic pH of 4.5–5.5 [9], it can cleave double-stranded DNA up to pH 7.0 and can make single-strand nicks in DNA up to pH 7.5 [10].

Earlier apoptosis studies supported the involvement of DNase II in apoptotic DNA cleavage in some models [11]. It was proposed that this nuclease could act as the apoptotic executioner nuclease and digest the nuclear chromatin [10, 11]. The general link of DNase II to apoptosis was further strengthened when its homologue NUC-1 was implicated in apoptotic DNA fragmentation in *C. elegans*. The NUC-1 enzyme, as well as DNase II, was also shown to be essential for engulfment-mediated DNA degradation [12, 13]. However, later studies shifted their focus from DNase II to caspase-activated and other apoptotic pathways; thus, the role of DNase II in apoptosis execution remains unconfirmed.

Recently, DNase II started attracting attention again, but this time due to its confirmed role in the newer cell death mechanisms, such as those triggered by permeabilized lysosomal membranes. The new data shows that lysosomal membrane permeabilization and the release of DNase II are central in the execution of several types of programmed non-apoptotic cell death [14, 15]. The term “DNase II-dependent cell death” is now being applied to some of these processes [15].

However, in order to implicate DNase II in cell death resulting from permeabilized lysosomes, it is necessary to demonstrate that after its release the enzyme in fact cleaves nuclear DNA. Therefore, the immunohistochemical detection of DNase II is not informative on its own, because it does not establish whether the nuclease is merely present or actively digesting DNA.

Here, we present a technique that resolves this limitation by labeling specific DNase II-type cleavage. The detection is a part of a dual labeling approach that simultaneously labels both the DNase II-type breaks and cathepsin D protease in fixed sections.

1.3 DNA Breaks of DNase I- and DNase II-Types

DNase II produces characteristic DNA breaks, so its enzymatic action on DNA can be determined even in fixed cells. DNase II catalyzes cleavage of cellular DNA to yield the breaks with 3'PO₄/5'OH DNA ends, which are called DNase II-type breaks [16–18]. These breaks differ from the 3'OH/5'PO₄ cuts produced by DNase I (and the majority of apoptotic nucleases including caspase-activated), because PO₄ and OH active groups at their terminals are inverted. The 3'OH/5'PO₄ DNA breaks are referred to as DNase I-type breaks [19]. The techniques that are mostly used to detect DNA breaks in fixed apoptotic cells, such as TUNEL or in situ ligation, both label only DNase I-type breaks [20, 21]. The DNase II-type breaks can be labeled by topoisomerase-based approaches [16–19, 22]. Their detection is mostly used to visualize phagolysosomes in macrophages clearing apoptotic cells [16].

1.4 Dual Detection Procedure

The dual detection combines labeling of DNase II-type breaks by vaccinia topoisomerase (VACC TOPO) and immunohistochemical detection of cathepsin D. In this procedure topoisomerase-based labeling of DNase II-type breaks is performed first, followed by the cathepsin D detection.

As a part of the dual labeling procedure, the VACC TOPO labeling protocol is performed differently in comparison to its mono-labeling variants. Its peculiar feature in this case is that it requires a biotin-labeled probe (vs. the directly labeled probe used before [16–19]) and the subsequent enzymatic amplification of its signal by the TSA system. This is done to counteract the signal decrease in DNA breaks detection, resulting from the processing of sections during the subsequent cathepsin D labeling step.

2 Materials

1. 5–6 µm-thick sections cut from formaldehyde-fixed, paraffin-embedded tissue blocks. Use slide brands that retain sections well, such as charged and precleaned slides.
2. Slide Brite clearing agent—xylene substitute (Sasco Chemical Group Albany, GA).
3. 80 % and 96 % Ethanol.
4. Oligoprobes 1 and 2—Hairpin-shaped biotin labeled oligonucleotide with a 12-base stability adapter oligo. PAGE or HPLC purification is recommended. Dilute with bidistilled water to 100 pmol/µL stock concentration. Combine in a 1:1 ratio to form Detection Probe.

Oligo 1: 5'-AAG GGA CCT GCB GCA GGT CCC TTG ATA CGA TTC TA-3'.

Oligo 2: 5'-TAG AAT CGT ATC-3'.

B—biotin-dT.

5. TSA-biotin enhancement kit (PerkinElmer) (*see Note 2*).
6. Vaccinia DNA topoisomerase 1—6 pmol/μL stock Vivid Technologies, Inc.
7. 50 mM Tris-HCl, pH 7.4
8. DNase II—500 ng/μL in the buffer supplied with the enzyme (Sigma Chemicals, St. Louis, MO). DNase II can be used in controls if verification of labeling specificity is required. Make the DNase II stock by diluting DNase II powder in water to the concentration of 1 mg/mL, aliquot in small volumes, and keep the stock at −20 °C. To run a reaction, dilute the stock solution 1:1 with the DNase II reaction buffer supplied with the enzyme (*see Note 3*).
9. Sodium citrate buffer (10 mM Sodium citrate, 0.05 % Tween 20, pH 6.0). Combine 2.94 g of Tri-sodium citrate (dihydrate) with 1 L of distilled water. Mix to dissolve. Adjust pH to 6.0 with 1 N HCl. Add 0.5 mL Tween 20 and mix well. Store at room temperature for 3 months or at 4 °C for longer storage.
10. Anti-Cathepsin D antibody; polyclonal rabbit Anti-Cathepsin D rat/human ab826 (Abcam).
11. Secondary antibody, donkey Anti-rabbit Alexa Fluor 568; Cat. # A10042 (Life Technologies).
12. 5% Donkey serum in PBS.
13. Phosphate-buffered saline (1× PBS): dissolve 9 g NaCl, 2.76 g NaH₂PO₄·H₂O, 5.56 g Na₂HPO₄·7H₂O in 800 mL of distilled water. Adjust to pH 7.4 with NaOH, and fill to 1 L with distilled water.
14. Vectashield with DAPI (Vector Laboratories, Burlingame, CA).
15. 22 × 22 mm or 22 × 40 mm glass and plastic coverslips (*see Note 4*).
16. Fluorescent microscope with appropriate filters and objectives.

3 Methods

1. Place the sections in a slide rack and dewax in Slide Brite clearing agent for 15 min (*see Note 5*), transfer to a fresh Slide Brite bath for an additional 5 min (*see Note 2*).
2. Rehydrate by passing through graded ethanol concentrations: 96 % Ethanol—2 × 5 min; 80 % Ethanol—5 min; water—2 × 5 min (*see Note 6*).
3. While sections are rinsed in water, combine in a vial: 100 pmoles of Detection Probe and 100 pmoles (3.3 μg) of vaccinia topoisomerase I in solution of 50 mM Tris-HCl, pH 7.4. Incubate at room temperature for 15 min to allow for probe activation. Use 25 μL of this reaction solution per section.
4. Aspirate water from sections and apply the reaction solution containing the Detection Probe and vaccinia topoisomerase I.

5. Incubate for 15 min at room temperature (23 °C) in a humidified chamber with a plastic coverslip.
6. Remove coverslips by gently immersing the slides vertically in a coplin jar containing water at room temperature. Then wash section 3 × 5 min in distilled water.
7. Add TNB blocking buffer and incubate slides in a humidified chamber for 30 min at room temperature (23 °C) (*see* Note 2).
8. Aspirate TNB blocking buffer and add 1:100 dilution of streptavidin-horseradish peroxidase conjugates (SA-HRP) in TNB blocking buffer. Incubate slides with SA-HRP for 30 min at room temperature. Use adequate reagent volume to cover the tissue section, generally 100 µL per section.
9. Prepare a 1:50 dilution of fluorophore tyramide stock in 1× Amplification Diluent from the kit. Pipette the fluorophore tyramide working solution onto each slide. Use enough working solution to completely cover the tissue section, generally 100 µL per section. Make fluorophore tyramide working solution immediately before each labeling. The solution cannot be reused, so discard any unused portion of it after labeling.
10. Incubate the slides at room temperature for 3–10 min. Monitor fluorescence increase under microscope until the first hints of structures can be seen. Quickly proceed to wash step.
11. Wash in TNT wash buffer for 3 × 5 min.
12. Place slides in Sodium Citrate buffer and microwave for 10 min. Let it stand for 5 min. Transfer to distilled water wash. Allow to cool to room temperature.
13. Pre-block section with 5 % donkey serum in PBS at room temperature for 20 min.
14. Incubate with primary antibody for Cathepsin D diluted 1:50 in 2 % donkey serum in PBS at 4 °C overnight.
15. Wash in PBS for 3 × 5 min.
16. Incubate with Alexa Fluor 568 donkey anti-rabbit IgG diluted 1:250 in 2 % donkey serum in PBS at room temperature for 1 h.
17. Wash in PBS for 3 × 5 min.
18. Cover section with an antifading solution such as Vectashield with DAPI, coverslip and analyze the signal using a fluorescent microscope. Double-strand DNA breaks with 5'OH will fluoresce green and antibody labeled cathepsin D will fluorescence red (Fig. 1).

4 Notes

1. Lysosomal DNase II (DNase IIoc) is by far the most likely source of blunt-ended 3P/5'OH DNA breaks, such as those detected by the VACC TOPO technique,

presented here. Nevertheless, the additional sources cannot be completely excluded. The alternative nuclease capable of producing this type of cleavage is DNase II-like acid DNase (DLAD), also known as DNase II β [23]. However, DLAD is expressed only in a few tissues. It is strongly expressed in the lens where it digests nuclear DNA during terminal differentiation of lens fiber cells [24]. Another potential source is leukocyte elastase inhibitor (LEI)-derived DNase II or L-DNase II, which is generated by proteolytic processing of serpinB1 [25]. L-DNase II has been implicated in some apoptotic mechanisms [26]. Although it produces breaks with terminal 3'P and 5'OH, its ability to cut double-stranded DNA generating blunt-ended DNA breaks, such as those detected by the VACC TOPO assay, has never been demonstrated.

2. Prepare components for Tyramide Signal Amplification (TSA) system before starting the labeling reaction. Although the TSA-based enhancement is used in a later stage of labeling, preparing for this reaction takes more than an hour. Therefore, make the TSA reagents before starting the labeling.
 - a. Make fluorophore tyramide stock solution. Use the fluorophore tyramide reagent supplied in individual vials with the TSA labeling kit. Add 300 μ L DMSO to the tyramide reagent vial. Fluorophore tyramide stock solution is stable for at least 3 months when stored at 4 °C.
 - b. Make TNT wash buffer containing: 0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.05 % Tween-20. If needed 0.3 % Triton X-100 can be substituted for the 0.05 % Tween-20. Alternatively PBS may be used as wash buffer.
 - c. Make TNB blocking buffer containing: 0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5 % Blocking Reagent (supplied with kit). To completely dissolve the blocking reagent, slowly add it in small increments to the buffer while stirring. Heat this solution gradually to 55 °C with continuous stirring. This can take 30–60 min. The solution will appear milky. Bring to room temperature before using. Aliquot and store at –20 °C for long-term use.
3. DNase II is used to produce positive controls. For this, use sections of normal tissues, such as normal bovine adrenal or rat heart. As additional controls to evaluate nonspecific background staining, perform mock reactions without enzymes.
4. Plastic coverslips are preferable for use during processing, as they are easier to remove from the section. Glass coverslips are used after final labeling for microscope viewing.
5. Alternatively, sections can be dewaxed in xylene for the same time. We use the xylene substitute Slide Brite because it produces the same result and is less toxic.
6. The standard VACC TOPO labeling procedure includes the Proteinase K digestion step performed after the rehydration (graded ethanol to water) step [16]. This is done to increase the VACC TOPO signal. However, it is not

essential with TSA enhancement and can be omitted. This step is not recommended in the case of dual labeling, as it can destroy cathepsin D.

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Fig. 1.

Experimental stroke 48 h after ischemia onset: dual detection of cathepsin D and DNase II-cleaved DNA. Proteolytic hydrolase cath D (red) and the specific nucleolytic activity of DNase II (green) are localized in endolysosomes in cellular cytoplasm. They are unevenly distributed between different cells and cytoplasmic sites. DAPI—cellular nuclei (blue). The diagram image on the right illustrates relative localizations of cath D and DNase II activity. Experimental stroke reproduced and sections prepared as described [27, 28]. Scale bar—25 μ m